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Sigma Proteins: Evolution of the Concept of Sigma Receptors

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Sigma Proteins: Evolution of the Concept of Sigma Receptors

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Preface

Pharmacology has evolved dramatically over the past several decades. Classical approaches were typically observational, using a range of bioassays and behavioral methods. While those approaches are still useful, today the field has moved into the molecular era as the molecular biology revolution and structural biology has shown us targets at the atomic level. The sigma receptors illustrate this evolution.

Sigma receptors were first described by Martin in 1976, based upon the unique constellation of actions of the benzomorphan SKF10,047 in the dog. Initially Martin classified them as opioid receptors based upon their reversal by the antagonist naloxone. Although not specified, Martin was likely using racemic (±) SKF10,047. This is important since only the (–)isomer has affinity for opioid receptors and was probably responsible for the naloxone reversibility he observed. With the resolution of the isomers, investigators redefined the $sigma_1$ receptor with the (+)isomer as a non-opioid receptor with a wide range of activities. A vast repertoire of agonists and antagonists were developed and $sigma_1$ binding sites were extensively described. However, in many respects sigma receptors remained an enigma. The cloning of the sigma1 receptor subtype (Sigma1) in 1996 was a major step forward in our understanding of the protein, culminating with the recent report of its crystal structure. Antisense knockdown approaches revealed effects similar to those of antagonists, confirming the selectivity of many of their actions and supporting their classification. Yet, many questions remain. While the protein has functionally active binding sites, its classification as a "receptor" is in question, since it is structurally unrelated to any of the established receptor families and has no intrinsic transduction mechanism. Rather, its physiological and pharmacological activities appear to result from its modulation of other proteins.

Sigma1 has been associated with a vast range of physiological effects and pathological processes, making it is difficult to provide an integrated assessment of Sigma1 action. There is strong evidence for its chaperone role in the endoplasmic reticulum, but equally strong evidence illustrating its modulatory role on receptors and channels, as well as a host of intracellular processes. It physically associates with G-protein-coupled receptors, ion channels, and a large range of other proteins, raising the possibility that it functions as a structural protein, possibly a scaffolding protein. Yet, it is not inert since occupancy of specific recognition sites within the protein has profound effects on a number of systems. Integrating the wide range of

activities has been difficult. Despite the marked advances in the field, we are left with many important questions to resolve.

This volume presents a timely summary of key perspectives on Sigma1 structure and function in a set of physiological and pathophysiological contexts. However, it remains a work in progress. It will be most interesting to see where the field moves in the next decade.

We thank Dr. James Barrett, Editor in Chief of the *Handbook of Experimental Pharmacology*, for contacting us to prepare this volume. We thank Susanne Dathe, Balamurugan Elumalai and Gayathri Silembarasan of Springer Press for overseeing the production and publication of this volume. Finally, we thank all of the Sigma researchers who have persevered over the decades and have collectively contributed to a renaissance of this field.

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Introduction to Sigma Proteins: Evolution of the Concept of Sigma Receptors

Felix J. Kim

Contents

Abstract

For over 40 years, scientists have endeavored to understand the so-called sigma receptors. During this time, the concept of sigma receptors has continuously and significantly evolved. With thousands of publications on the subject, these proteins have been implicated in various diseases, disorders, and physiological processes. Nevertheless, we are just beginning to understand what sigma proteins do and how they work. Two subtypes have been identified, Sigma1 and Sigma2. Whereas Sigma1 (also known as sigma-1 receptor, Sig1R, σ1 receptor, and several other names) was cloned over 20 years ago, Sigma2 (sigma-2 receptor, σ2 receptor) was cloned very recently and had remained a pharmacologically defined entity. In this volume, we will focus primarily on Sigma1. We will highlight

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several key subject areas in which Sigma1 has been well characterized as well as (re)emerging areas of interest. Despite the large number of publications regarding Sigma1, several fundamental questions remain unanswered or only partially answered. Most of what we know about Sigma1 comes from pharmacological studies; however, a clearly defined molecular mechanism of action remains elusive. One concept has become clear; Sigma1 is not a traditional receptor. Sigma1 is now considered a unique pharmacologically regulated integral membrane chaperone or scaffolding protein. A number of landmark discoveries over the past decade have begun to reshape the concept of sigma receptors. With the rapid emergence of new information, development of new tools, and changing conceptual frameworks, the field is poised for a period of accelerated progress.

Keywords

Alcohol abuse • Allosteric modulation • Anchor patch • Cancer • Chaperone • Crystal structure • Drug addiction • Drug mechanism of action • Imaging agents • Medicinal chemistry • Neurodegeneration • Neuronal excitability • Nuclear magnetic resonance • Pain • Pharmacology • Puzzle • Scaffold • Self-administration • Sigma1 • Sigma-1 receptor • Sigma2 • Sigma-2 receptor • Small molecule modulator • Three-dimensional homology model

1 Historical Perspective

The concept of sigma receptors has continually evolved for over four decades. While most in the field agree that they are important, there is little agreement on anything else. Even the nomenclature to describe the binding sites varies. In the literature, one will find: σ_1 receptor, σ1R, σ_2 receptor, sigma1 receptor, sigma2 receptor, sigma-1 receptor, sigma-2 receptor, $sigma_1$ receptor, $sigma_2$ receptor, Sig-1R, Sigma-R1, SigmaR1, Sigmar1, ALS16, AAG8, and Sigma1 (which is our preferred nomenclature for this subtype, to indicate that this unique protein is not a traditional receptor).

The story has undergone many twists and turns, and every decade since its original identification, major new developments and discoveries have attempted to redefine the field. Originally identified in 1976, Martin and colleagues proposed three distinct opioid receptor classes, mu, kappa, and sigma, based upon behavioral studies using morphine, ketocyclazocine, and the benzomorphan SKF10047. They noted that the opioid antagonist naltrexone antagonized them all, leading to the identification of sigma as an opioid receptor (Martin et al. 1976). In the original study, the SKF10047 stereoisomer used was not described; however, subsequent investigators used (+)-SKF10047 to define sigma binding sites and identified them as receptors that clearly were not opioid (Su 1982). Since then, a large number of chemically diverse compounds that have affinity for sigma receptors have been reported (reviewed in the chapter by Weber and Wunsch in this volume and more broadly reviewed, collectively in Cobos et al. 2008; Maurice and Su 2009; Narayanan et al. 2011). As more compounds with affinity for sigma binding sites became

available, the putative sigma receptors were subdivided into two categories: Sigma1 and Sigma2 based primarily on ligand binding studies (Hellewell and Bowen 1990).

The cloning of Sigma1 (Hanner et al. 1996) was a major milestone in the field. It revealed that Sigma1 was unlike any traditional receptor; indeed, it later became clear that Sigma1 shares no significant homology with any other protein encoded in the human genome. As the field advanced through the end of the twentieth and into the twenty-first century, although the label "receptor" continued (and continues) to be used, it became increasingly clear that Sigma1 does not fit the traditional definition of a receptor. In 2007, the notion that Sigma1 is not a receptor, but rather, a chaperone protein was introduced (Hayashi and Su 2007), and the notion that Sigma1 functions as an oligomeric structure was introduced in 2014 (Gromek et al. 2014). A three-dimensional (3-D) homology model was developed in 2011 (Laurini et al. 2011) and a solution phase structure was established by nuclear magnetic resonance (NMR) in 2015 (Ortega-Roldan et al. 2015). In 2016, the first crystal structure of Sigma1 was resolved (Schmidt et al. 2016). Very recently Alon and colleagues published the cloning of Sigma2 (Alon et al. 2017; Kim and Pasternak 2017). The crystal structure of Sigma2 is surely not far behind.

Functional studies in rodent models of behavior and in vitro cell based assays have implicated both subtypes of sigma receptor in a range of physiological and patho-physiological contexts: neurodegenerative diseases, neuronal plasticity, neuronal development, cognition, memory, learning, various types of pain, cancer, immune modulation, and many others. However, despite more than 4,000 publications on the subject over four decades, fundamental questions regarding what sigma proteins do in each context and how they work are unanswered or partially answered. This historical overview does not cover the hundreds of important discoveries and nuances of the twists and turns underlying the evolution of the field. A detailed and comprehensive history of sigma proteins would require multiple volumes from diverse perspectives. This volume will focus primarily on the literature regarding Sigma1 and will provide insights into the state of the field through a number of key examples.

2 Objectives of this Volume

So, what is the state of the field? The analogy of a puzzle comes to mind, and we see the more than 4,000 publications representing pieces of a complex jigsaw puzzle. Thus, our goal is to identify "anchor patches" of the Sigma1 puzzle, that is, key related findings that provide disproportionate insight into the structure and function of Sigma1 (concept of "anchor patches" reviewed in Cho et al. 2010). These include our current understanding of: Sigma1 structural biology; Sigma1 pharmacology; Sigma1 in neurodegeneration and neuronal plasticity; Sigma1 in cancer and its ligands in the context of cancer; Sigma protein radiotracers and imaging agents; Sigma1 in pain; Sigma1 ligands as non-opioid antinociceptive agents; and Sigma1 in drug abuse and addiction. This introduction to the volume *Sigma Proteins:*

Evolution of the Concept of Sigma Receptors provides a brief overview of the chapters that address some of the "anchor patches" to the sigma puzzle.

3 Insights into the Structure of Sigma1

For decades, sigma proteins have remained enigmatic binding sites, largely defined by the pharmacology of small molecule ligands. Recently, important advances have been made, which provide significant insight into our understanding of the structure and functionality of Sigma1. Perhaps the most exciting recent development has been the publication of the crystal structure of Sigma1 (Schmidt et al. 2016; Kruse 2016). The chapter by Kruse and colleagues entitled *Structural Insights into Sigma1 Function* discusses their Sigma1 crystallographic studies and the technological innovations that enabled this landmark discovery. They propose that the crystal structure provides a framework to understand the published mutagenesis data and the diverse molecular pharmacology of Sigma1 ligands (Kruse 2016). The crystal structure shows a high resolution but static snapshot of Sigma1. With this information now available, it will be fascinating to investigate and elucidate the differential dynamic effects of Sigma1 antagonists/inhibitors, agonists/activators, and membrane lipids/cholesterols on its conformation and subsequent modulation of Sigma1 associated proteins. The development of nuclear magnetic resonance (NMR) approaches and in silico tools to analyze the structural biology and pharmacology of Sigma1 represents another major advance in the field. The *3-D homology model of Sigma1 receptor* chapter by Pricl and colleagues proposes a three-dimensional (3-D) model of Sigma1 generated from homology modeling techniques, and proposes how this approach can be applied as a docking model-based virtual drug screen for rational ligand design (Laurini et al. 2017).

Interestingly, the crystal structure and 3-D homology models do not match. For example, whereas previous biochemical studies proposed a two-transmembrane domain topology, the crystal structure shows a single transmembrane domain protein with a short ER-lumenal peptide and one relatively large cytoplasmic domain containing a cupin-like ligand-binding barrel (Schmidt et al. 2016; Kruse 2016). According to Pricl, this discrepancy raises a critical question: which is the true structure of Sigma1, the NMR solution-solved and in silico derived 3-D homology model or the X-ray-solved crystal structure? How can the differences between the two structures be explained? Pricl and colleagues propose that the Sigma1 protein may adopt different structures under solid (revealed by the crystal structure) and solution (revealed by the in silico and NMR models) states. Ultimately, Pricl and colleagues argue that the field still has a long way to go before it is able to provide an unequivocal answer to these questions. Resolution of these discrepancies and advances in our understanding of Sigma1 structure will position the field for new discoveries as well as re-evaluation of older data and models.

4 Development of Sigma1 Medicinal Chemistry

Perhaps one of the most daunting tasks in producing this volume was to provide clarity and coherence to the vast, diverse, and complex field of Sigma1 medicinal chemistry. Weber and Wunsch accept this challenge in their chapter entitled *Medicinal Chemistry of Sigma1 (σ_1) Receptor Ligands: Pharmacophore Models, Synthesis, Structure Affinity Relationships, and Pharmacological Approaches* (Weber and Wunsch 2017). This chapter comprises two principal parts: (1) review of the various pharmacophore models for Sigma1 ligands and the role of the 3-D homology model and the crystal structure in future development; (2) the synthesis and biological properties of nine prototypic Sigma1 ligands. In addition to experimentally determined ligand binding affinity and molecular dynamics simulations based on a 3-D homology model, the authors present the following published data as readouts of biological properties: cancer cell growth and survival inhibition and apoptosis, neurite outgrowth in vitro, and data from pain behavioral assays. The authors also review the discovery and development of Sigma1 ligands as radiotracers for positron emission tomography (PET) and imaging agents, including an agent that is in clinical trials for central nervous system (CNS) imaging of patients suffering from major depression.

During the past decade, considerable progress has been made, and Weber and Wunsch present an encouraging outlook for the evolution of Sigma1 medicinal chemistry in light of recent structural discoveries as well as the advancement of a Sigma1 ligand, S1RA, through clinical trials for neuropathic pain (also discussed in the chapters by Vela and colleagues (Merlos et al. 2017)).

5 Sigma1 Pharmacology in Neurodegeneration and Neuronal Excitability

The vast majority of the Sigma1 literature addresses aspects of neuropharmacology. In their chapter *Sigma1 (σ_1) Receptor in Memory and Neurodegenerative Diseases*, Maurice and Goguadze review and discuss pharmacologic and genetic evidence of Sigma1 involvement in learning and memory disorders, cognitive impairment, and neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and Huntington's disease (Maurice and Goguadze 2017). They review a number of recent publications that highlight the efficacy of drugs with affinity for Sigma1 in mitigating symptoms associated with neurodegenerative disorders in preclinical rodent models. They also point out that compounds with affinity for Sigma1 are in clinical trials, and that one Sigma1 drug is in phase II clinical trials for Alzheimer's disease (Maurice and Goguadze 2017).

Sigma1 in neuronal signaling and regulation of ion channels is reviewed in the chapter by Kourrich entitled *Ion Channels and Neuronal Excitability* (Kourrich 2017). In this chapter, Kourrich describes and discusses Sigma1 dependent modulation of voltage gated ion channels (VGICs) and ligand gated ion channels (LGICs). He describes the range of proteins with which Sigma1 has been reported

to interact and proposes this as the reason for the plethora of neuronal functions in which Sigma1 has been implicated. Kourrich proposes that Sigma1 is an integral membrane protein at the plasma membrane, with extracellular N- and C-termini that regulates VGIC conductance at the cell surface, and proposes that Sigma1 is an atypical auxiliary regulatory subunit for ion channels for VGICs. He discusses the potential mechanisms to explain observed effects of Sigma1-associated activities on intrinsic and synaptic excitability, and how these mechanisms affect overall neuronal activity.

6 Sigma1 as a Drug Target for Pain

As Vela and colleagues emphasize in their chapter, *Sigma-1 Receptor and Pain*, there is a critical need for new potent and efficacious non-opioid analgesics or agents that increase the potency and efficacy of opioids in order to diminish or bypass their addictive properties and other serious, unwanted side effects (Merlos et al. 2017).

The authors review the literature as well as their own studies demonstrating the roles of Sigma1 in nociception. They discuss the pain-attenuated phenotype of the published *SIGMAR1* knockout mouse and the antinociceptive properties of Sigma1 putative antagonists/inhibitors in pain of varied etiology, including neuropathic, inflammatory, ischemic, visceral, and postoperative pain. They review the proposed mechanisms by which Sigma1 antagonists/inhibitors elicit antinociceptive effects in peripheral as well as central nervous system (central) pain. They propose that unlike opioids, Sigma1 antagonists/inhibitors do not alter normal sensory perception or mechanical and thermal sensitivity thresholds in normal animals but only exert antihyperalgesic and antiallodynic effects specifically under sensitizing or pathophysiological conditions such as chronic pain. The authors point out that Sigma1 antagonists/inhibitors are thus not analgesics, as strictly defined, but rather antiallodynic and antihyperalgesic agents.

Finally, Vela and colleagues highlight and describe in detail S1RA (also known as E-52862), an investigational Sigma1 antagonists/inhibitors currently in phase II clinical trials for chronic neuropathic pain and postoperative pain in combination with morphine. Clearly, the outcome of these clinical trials is of considerable interest to the field as it will confirm the potential of this new target, drug class, and approach to pain management.

Sigma1 has been associated with myriad signaling and transduction systems over the decades. Evidence over the past 20 years has demonstrated that Sigma1 ligands can modulate opioid analgesia in vivo and opioid receptor signaling mechanisms in vitro. In his chapter entitled *Allosteric Modulation of Opioid G-Protein Coupled Receptors by Sigma$_1$ Receptors*, Pasternak describes how Sigma1 ligands can function as allosteric modulators of G-protein coupled receptors (GPCR) function through their association with the Sigma1 (Pasternak 2017). He reviews the literature for evidence of the signal modulatory role of

Sigma1 on GPCR activity in various regions of the CNS and argues that the general actions of Sigma1 extend beyond its putative chaperone actions.

Sigma1 antagonist/inhibitor potentiation of opioid analgesia highlights their potential use in combination with opioids, as opioid adjuvants, to selectively enhance analgesic effects while minimizing the dose of opioids, thus reducing side effects and potential for addiction and increasing the safety margin of opioid treatments. These data along with the antinociceptive properties of S1RA alone represent a promising new approach to safely treat intractable chronic pain conditions. Emergence of Sigma1 ligands as novel, non-opioid pain relief agent is timely indeed, in light of the current opioid epidemic in the USA.

7 Alcohol and Drug Abuse and Addiction

Sigma proteins, both Sigma1 and Sigma2, historically have been described as modulators of the effects of psychomotor stimulants, such as cocaine and methamphetamine, and have been proposed as agents to mitigate stimulant drug abuse. However, the published results have been varied and the pharmacological mechanisms underlying these effects remain unclear. Katz and colleagues in their chapter, *A Role for Sigma Receptors in Stimulant Self-Administration and Addiction*, review the effects of sigma receptor ligands (both putative agonists/activators and antagonists/inhibitors) in three relevant pharmacological assays of rodent behavior: stimulant discrimination, place-conditioning, and self-administration (Katz et al. 2017). The literature suggests that Sigma1 agonists/activators generally substitute for psychomotor stimulants in the discrimination assay, and Sigma1 antagonists/inhibitors generally block stimulant effects in the place-conditioning assay. However, the responses are more complex and do not necessarily follow these general trends, and appear to be condition and context dependent. Interestingly, test subjects self-administered Sigma1 agonists/activators only after stimulant self-administration, suggesting that psychostimulants modify the status of Sigma1 in a manner that creates independent reinforcement mechanisms. The authors observe that selective Sigma1 antagonists/inhibitors do not block stimulant self-administration; however, nonselective Sigma1 antagonists/inhibitors that also bind the dopamine transporter can decrease stimulant self-administration. Thus, they propose that concomitant targeting of both dopaminergic and sigma receptors selectively suppresses mechanisms involved in stimulant abuse and reveal the possibility of new drug combination strategies to prevent stimulant abuse.

Whereas extensive research has been performed regarding the neurobiological mechanisms underlying alcohol addiction, pharmacological intervention in alcohol abuse disorders remains limited and ultimately ineffective. In the chapter *Sigma Receptors and Alcohol Use Disorders*, Sabino and Cottone review emerging evidence suggesting that Sigma1 plays a role in the rewarding and reinforcing effects of alcohol, and that Sigma1 may be a novel target for the pharmacological treatment of alcohol use disorders (Sabino and Cottone 2016). This work builds upon established literature implicating Sigma1 in psychostimulant pharmacology. The

authors review the literature describing the efficacy of Sigma1 antagonists/inhibitors in reducing excessive alcohol drinking and alcohol-seeking behavior in several animal models.

8 Sigma1 Pharmacology in the Context of Cancer

Most of the literature regarding Sigma1 describes it in the context of neuropharmacology; however, a number of publications over the years have suggested a role for Sigma1 in tumor biology. Although there is currently no clinically used anticancer drug that targets Sigma1, a growing body of evidence supports the potential of Sigma1 ligands as cancer therapeutic agents with a range of beneficial activities. Indeed, in preclinical models, compounds with affinity for Sigma1 have been reported to inhibit cancer cell proliferation and survival, tumor growth, cell adhesion and migration, to alleviate cancer-associated pain, and to have immunomodulatory properties. In their chapter *Sigma1 Pharmacology in the Context of Cancer*, Kim and Maher review and discuss the status of Sigma1 in cancer (Kim and Maher 2017).

The authors point out that although the literature supports a potential role for Sigma1 in cancer, fundamental questions regarding the pharmacological mechanism of action of Sigma1 ligands and the physiological relevance of aberrant *SIGMAR1* transcript and Sigma1 protein expression in certain cancers remain unanswered or only partially answered. For example, there is no compelling evidence that *SIGMAR1* is an oncogene or that Sigma1 is an oncogenic driver protein; however, several studies have demonstrated that cancer cells require functional, intact Sigma1 to grow, proliferate, and survive. Kim and Maher propose and provide preliminary direct and indirect evidence in support of the hypothesis that Sigma1 is a component of the cancer cell support machinery promoting protein and lipid homeostasis, that it facilitates protein interaction networks, and that it allosterically modulates the activity of its associated proteins. The authors propose that Sigma1 ligands may be allosteric modulators of protein–protein interactions. This is consistent with the prevailing but unclearly defined notion that Sigma1 itself is devoid of intrinsic signaling or enzymatic activity, rather it acts as a modulator of the intracellular signaling and activities of other receptor systems. However, the biochemical mechanism by which Sigma1 elicits these effects remains unclear. Recent developments in Sigma1 structural biology should facilitate progress in this domain.

9 Sigma2 as a Target for Imaging Agents

Mach and colleagues review Sigma2 ligands as imaging tools in their chapter *Molecular Probes for Imaging the Sigma-2 Receptor:* In Vitro *and* In Vivo *Imaging Studies* (Zeng et al. 2017). The sigma-2 (σ_2) receptor or Sigma2 has been a pharmacologically defined entity for decades. Interestingly, studies with

radiolabeled probes have demonstrated that the level of Sigma2 binding sites correlates with the proliferative status of solid tumors (Wheeler et al. 2000). Thus, small molecule radiotracers with affinity and selectivity for Sigma2 have been evaluated in preclinical and more recently in clinical trials to assess the proliferative status of human tumors by positron emission tomography (PET) (Zeng et al. 2017). Of note, the authors describe the development and promising results from preliminary clinical imaging studies with [^{18}F]ISO-1, a Sigma2 probe, in cancer patients.

The true utility of imaging Sigma2 in solid tumors as a diagnostic and/or predictive biomarker of therapeutic response will depend on a clearer understanding of what has remained an enigmatic pharmacological binding site until very recently. Since the writing of this volume, Sigma2 has been cloned and identified as transmembrane protein 97 (TMEM97), a relatively poorly understood integral membrane protein implicated in cholesterol metabolism (Alon et al. 2017; Kim and Pasternak 2017; Bartz et al. 2009). With the cloning of Sigma2/TMEM97, the compounds, radiotracers, and fluorescent probes developed for Sigma2 over the decades now have a biochemically defined target for pharmacological mechanism of action studies. And the field is poised to open another interesting new avenue of research.

10 Outlook

The sigma proteins have been primarily defined by the activities regulated by their ligands. In the case of Sigma1, it has no clearly defined signaling or enzymatic activity, and the pharmacology of Sigma1 ligands has been defined by the proteins with which it interacts. The myriad, context dependent effects of Sigma1 ligands present a complex picture. There is still much to be done to define unifying mechanisms of action of Sigma1 ligands. A fundamentally important question is what are the structural changes that define Sigma1 agonists/activators and antagonists/inhibitors, as these putative pharmacological activities have remained undefined at the molecular level. The structural insights and tools that have recently emerged will be instrumental in answering fundamental questions regarding how these proteins work, how ligands modulate their activity, and will accelerate drug discovery in this field.

The concept of the sigma receptor has evolved significantly over the past 40 years. Along the way, thousands of publications on the subject have provided key pieces to the sigma puzzle, and the field is in its best position yet to connect the puzzle pieces and to establish a clearer picture of the sigma proteins, how they work, and to explain their role in the diverse physiological and pathophysiological processes in which these proteins have been implicated.

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Structural Insights into Sigma1 Function

Andrew Kruse

Contents

Abstract

Sigma1 (also known as this sigma-1 receptor) is an unusual and enigmatic transmembrane protein implicated in a diverse array of biological processes ranging from neurodegenerative disease to cancer. Despite decades of research, the molecular architecture of Sigma1 is only beginning to become clear. Recent work has established that Sigma1 is an oligomer, and crystallographic studies have now offered the first high-resolution views of its molecular structure. For the first time, these results provide a detailed framework to understand mutagenesis data and the molecular pharmacology of Sigma1 ligands. Structural data also raise new questions surrounding the mechanisms of ligand activity and the molecular basis for interactions between Sigma1 and other proteins. As Sigma1 research enters the structural era, the field is poised for new discoveries and reevaluation of old data and old models.

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Crystal structure • Ligand binding • Oligomer • Sigma1 • Sigma-1 receptor • X-ray crystallography

1 Introduction

Sigma1 (also known as the sigma-1 receptor) is an unusual transmembrane receptor first discovered 40 years ago and subsequently studied extensively for its pharmacological properties, which in some respects resemble those of opioid receptors (Walker et al. 1990). However, the development of photoaffinity probes and enantiomerically pure ligands later showed that Sigma1 resemblance to opioid receptors is largely superficial. Unlike opioid receptors, Sigma1 has a molecular weight of ~25 kDa, and it shows a strong preference for the (+) enantiomers of benzomorphan ligands. In contrast, true opioid receptors selectively bind the (−) enantiomer (Martin et al. 1984). Moreover, the effects of Sigma1 ligands are not antagonized by the opioid antagonists naltrexone and naloxone (Largent et al. 1987), which block agonist activity at the μ, δ, and κ opioid receptors. Finally, unlike the opioid receptors, Sigma1 does not appear to signal through heterotrimeric G proteins (Lupardus et al. 2000), further confirming its lack of relation to opioid receptors.

While Sigma1 was extensively characterized with respect to its pharmacology in the 1980s and early 1990s, the first structural insights regarding Sigma1 came only in 1996 when the protein was cloned from guinea pig liver (Hanner et al. 1996), and shortly thereafter from a human cell line (Kekuda et al. 1996) as well as mouse (Seth et al. 1997) and rat tissues (Seth et al. 1998). Remarkably, the sequence of Sigma1 shows no similarity to any other mammalian protein, but it possesses clear homology (30% sequence identity) to the yeast C8–C7 sterol isomerase Erg2p. Hydrophobicity analysis of the guinea pig Sigma1 protein sequence showed a single highly hydrophobic segment with a potential ER-retention signal at its amino terminus, suggesting a single-pass transmembrane topology with a short luminal tail at the amino-terminus and the majority of the protein located on the cytosolic side of the ER membrane. As discussed below, this single-pass transmembrane model was less widely accepted than later proposed architectures placing both the amino- and carboxy-termini in the ER lumen, although the prediction of a single-pass fold was ultimately shown to be correct.

The determination of the receptor's primary sequence led to extensive prediction and speculation regarding the three-dimensional structure of Sigma1. One report (Aydar et al. 2002) proposed a two-pass transmembrane architecture on the basis of antibody staining with Sigma1 fused to amino- and carboxy-terminal green fluorescent protein (GFP). An important caveat to this work, however, is that GFP is known to be poorly secreted (Li et al. 2002), and the amino-terminal fusion may have altered the insertion topology of the protein. The two-pass model was widely accepted as established fact, serving as the basis for efforts to map the position of the putative second transmembrane domain (Ortega-Roldan et al. 2015) and other

studies using molecular modeling to predict the receptor's structure computationally (Laurini et al. 2011). In contrast, efforts to determine the Sigma1 structure experimentally lagged behind, hindered by the myriad challenges associated with biochemical manipulation of integral membrane proteins.

2 Crystal Structure of Human Sigma1

Beginning in 2007, a series of technological innovations revolutionized membrane protein crystallography, particularly for G protein-coupled receptors (GPCRs). Advances include the use of fusion proteins like T4 lysozyme (Rosenbaum et al. 2007), lipid mesophase crystallization techniques (Caffrey et al. 2012), microdiffraction (Smith et al. 2012), and novel detergents (Chae et al. 2010). Together, these methods have led to dozens of GPCR structures, and these approaches are increasingly finding application in membrane protein crystallography more generally.

To determine the structure of Sigma1, a GPCR-inspired approach was employed (Schmidt et al. 2016). The receptor was expressed using *Sf9* insect cells to produce high levels of functional protein, which was then purified using lauryl maltose neopentyl glycol (LMNG), a protein-stabilizing detergent that has been widely used in the GPCR field. This detergent has an exceptionally low critical micelle concentration (Chung et al. 2012), and it has been shown to enhance stability of monomeric membrane proteins as well as preserve the stability of multi-protein complexes (Chae et al. 2010).

Purification of the full-length Sigma1 protein was achieved by antibody affinity chromatography, allowing rapid isolation of Sigma1 in high biochemical purity (Schmidt et al. 2016). Purified protein was then crystallized using the lipidic cubic phase (LCP) method (Caffrey et al. 2012). This approach entails reconstituting the protein in a liquid crystalline lipid bilayer and performing crystallization experiments with Sigma1 embedded in this membrane throughout the crystallogenesis process. Consequently, LCP crystals typically show arrays of protein molecules in flat membrane bilayers stacked together to form the crystal lattice. In the case of Sigma1, this approach led to structures (Schmidt et al. 2016) of the receptor bound to two chemically distinct ligands: PD144418, a high-affinity, selective antagonist (Lever et al. 2014; Akunne et al. 1997), and 4-IBP, a high-affinity ligand with an incompletely understood efficacy profile (John et al. 1994). These ligands were chosen by screening a small collection of compounds with 1 nM or higher affinity in crystallization trials, with PD144418 and 4-IBP showing the largest improvements in crystal size and appearance. While Sigma1 structure agrees closely with prior pharmacological data, it shows little similarity to previous computational models (Laurini et al. 2011), highlighting the challenges of predicting membrane protein structure ab initio.

The overall structure of the protein is unusual, with no significant resemblance to any other protein of known structure. Contrary to the prevailing view that the receptor

possessed a two-pass transmembrane architecture, the structure reveals definitively that Sigma1 possesses only a single transmembrane domain, located at its amino terminus and encompassing residues 8 to 32. The remainder of the structure forms a single domain, with the ligand-binding site at its center. In the crystal, the receptor exists in a trimeric arrangement in which three protomers are intimately associated to form a flat triangle, with a transmembrane domain at each corner (Fig. 1 a, b). The entire membrane-proximal surface is flat and hydrophobic, and the presence of ordered lipid molecules indicates that this surface is likely buried within the membrane. The receptor thus contains structural elements typical of both transmembrane receptors and peripheral membrane proteins.

The carboxy-terminal cytosolic domain of Sigma1 comprises the bulk of the protein, encompassing residues from 33 to 223. The overall fold of this region is unlike any other protein crystallized to date, but at its core contains a cupin-like β-barrel which encloses the bound ligand (Fig. 2). The cupin fold is a conserved structural motif found in a wide variety of proteins, many of which are bacterial metalloenzymes. In most of these proteins, the barrel-like cupin fold is essential for binding to small-molecule substrates and catalytic metal ions (Dunwell et al. 2001). In most cases, these enzymes perform redox chemistry on small-molecule metabolites. In the case of Sigma1, this fold serves to envelope the ligand, occluding it entirely from solvent. The carboxy-terminal domain also contains the entire oligomerization interface, mediating the threefold non-crystallographic symmetry. Although each of the three protomers in the structure is crystallographically independent, they show no significant differences other than in orientation of the transmembrane domain. Lattice contacts are mostly mediated by these transmembrane domains, which pack in both parallel and antiparallel configurations to form the crystal.

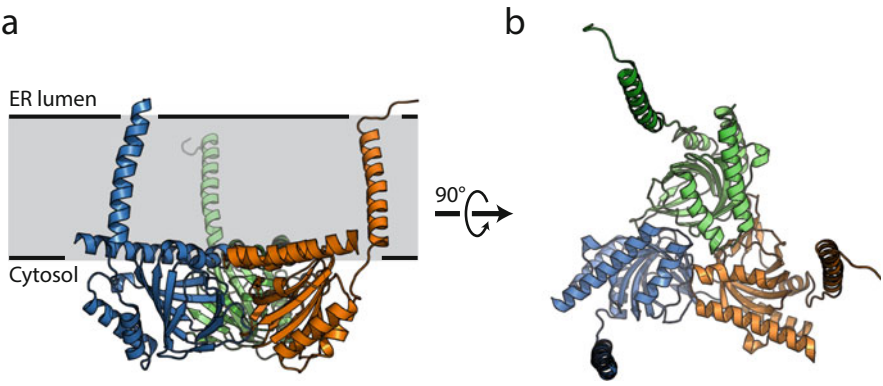

Fig. 1 Overall structure of Sigma1: The overall fold of Sigma1 is unusual, with three protomers forming a triangular structure with a single transmembrane helix at each corner. The majority of the protein is located carboxy-terminal to the transmembrane domain, on the cytoplasmic surface of the membrane. (**a**) The structure is shown viewed parallel to the membrane plane. (**b**) The triangular architecture of the receptor is apparent when viewed through the membrane plane facing the cytosol

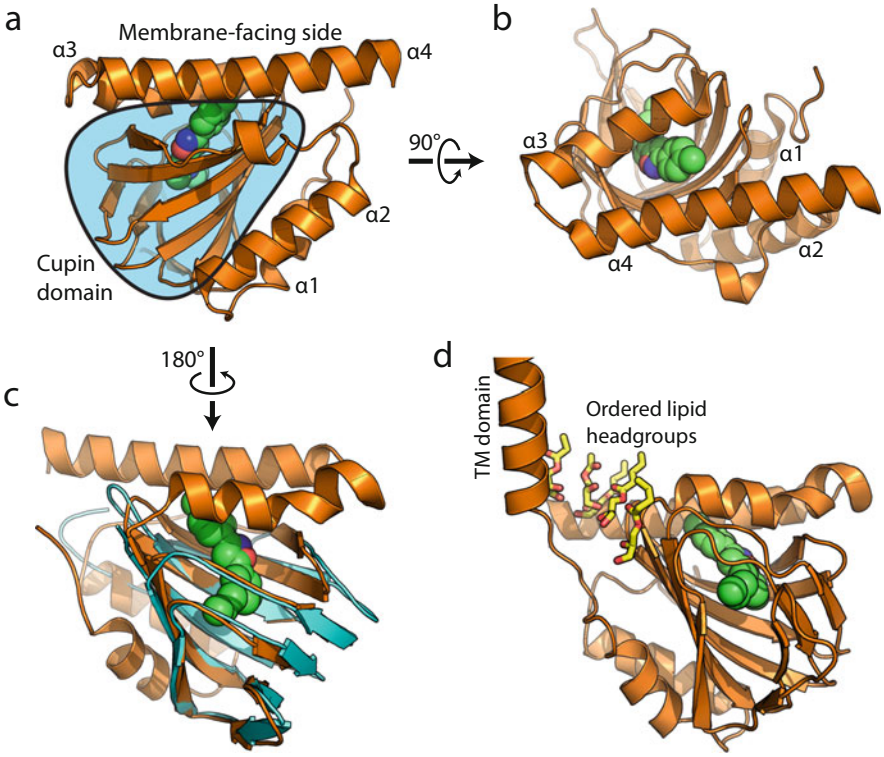

Fig. 2 Structure of the carboxy-terminal domain: The carboxy-terminal domain of Sigma1 makes up the bulk of the protein. (**a**) Viewed from the side (i.e., parallel to the membrane) the cupin domain at the core of the protein is shaded in *blue*. The bound antagonist PD144418 is shown in *spheres* at the center of the cupin domain. (**b**) Viewed from the top (membrane-facing side) the major structural elements are visible. (**c**) A bacterial cupin protein (*light blue*, PDB ID 3BCW) is superimposed on Sigma1, showing the high degree of structural conservation. (**d**) A cluster of ordered lipids are observed in the crystal structure at the junction of the carboxy-terminal domain and the transmembrane helix (shown in *yellow sticks*, oxygen atoms in *red*). The flexible tails of the lipids are not crystallographically resolved

3 Structural Basis for Ligand Recognition

The structures of Sigma1 bound to two drug-like ligands offer the first detailed views of ligand recognition by the receptor. The two structures are remarkably similar to one another, with few differences in ligand/receptor interactions (Schmidt et al. 2016). Previous work using site-directed mutagenesis and radioligand-binding assays identified many of the key residues that are essential for ligand-binding activity. Among these, Asp126 and Glu172 are particularly notable, as mutation of either results in a profound loss of ligand-binding activity (Seth et al. 2001). The crystal structures show

that Glu172 serves as a counterion to the protonated ligand amine, directly interacting via a hydrogen bond. Like Glu172, Asp126 is also essential for high-affinity ligand binding. Unlike Glu172 however, it does not interact directly with the ligand. Instead, Asp126 engages in a 2.6 Å hydrogen bond with Glu172, indicating that it must be protonated and resulting in overall charge neutrality in the binding pocket when the ligand is bound. With the exception of Asp126 and Glu172, the ligand-binding site is hydrophobic overall, and is largely composed of aromatic residues. Figure 3 shows the structure of the ligand-binding site, highlighting residues in contact with the bound ligand.

The solvent-occluded charge–charge interaction in the binding site closely resembles similar ligand-binding modes observed in biogenic amine GPCRs, including receptors for acetylcholine (Haga et al. 2012; Kruse et al. 2012), dopamine (Chien et al. 2010), histamine (Shimamura et al. 2011), and serotonin (Wang et al. 2013; Wacker et al. 2013). In these receptors, the conserved residue Asp3.32 (Ballesteros–Weinstein numbering (Ballesteros and Weinstein 1995)) engages in a salt bridge with the protonated ligand amine, paralleling the role of Glu172 in Sigma1. Although Sigma1 is unrelated to the aminergic GPCRs in sequence, the close parallels in binding site structure offer an explanation for the cross-reactivity of ligands like haloperidol, which binds both D_2 dopamine receptor and Sigma1 with nanomolar potency. It is possible that the similar binding modes arise from convergent evolution of biogenic amine recognition sites, which could imply that Sigma1 acts as a receptor for a neurotransmitter or similar small molecule. However, as discussed below it remains unknown if Sigma1 indeed responds to any endogenous agonist.

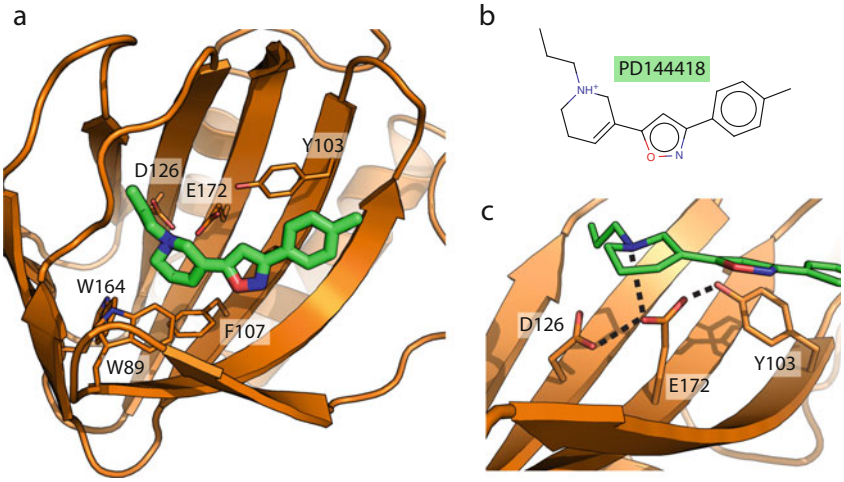

Fig. 3 Structure of the ligand-binding site: The ligand-binding site of Sigma1 is shown in two different views. (**a**) An overall view of the binding site reveals the largely hydrophobic contacts between the ligand and Sigma1, with the exception of a salt bridge to Glu172. (**b**) The chemical structure of the bound ligand PD144418. (**c**) A close-up of the binding site shows the extended polar network including Asp126 and Tyr103 as hydrogen-bonding partners to Glu172

Surprisingly, the ligand-binding site in both of the crystal structures is entirely occluded from solvent, offering no possible path for ligand entry or egress. This shows that the protein must be able to undergo dynamic changes to allow ligands to bind and dissociation from the receptor, but the path of ligand entry and exit remains unknown. The enclosed ligand-binding site offers a clear explanation for the very slow binding kinetics of most sigma ligands (Itzhak 1989), contrasting with opioid receptors which show rapid binding kinetics at their highly exposed ligand-binding sites (Cassel et al. 2005).

In addition to drug-like small molecules, Sigma1 has also been shown to bind to a range of lipids, including sterols like progesterone as well as sphingolipids (Ramachandran et al. 2009). Because crystallization experiments were conducted in the absence of these molecules the structural basis for their interaction with Sigma1 remains unknown. Nonetheless, a cluster of four bound monoolein lipid molecules are observed in the structure in a cleft between the transmembrane domain and the carboxy-terminal domain. This region is flanked by Gln33, Leu100, Trp121, Val177, and Leu214, and may serve as a site for lipid regulation of Sigma1 activity.

4 Oligomerization

Recent work from multiple labs has shown evidence that the Sigma1 receptor is likely to function as an oligomer, with possible regulation of oligomerization state by small-molecule ligands (Mishra et al. 2015; Gromek et al. 2014). The crystal structures further support this idea, showing an intimately associated trimer formed by the carboxy-terminal domain of each protomer. The interaction surface is extensive, involving more than 30 residues in each protomer, primarily along loops of the adjacent β-strands in the cupin domain, particularly along the cytosolic face (Fig. 4a). The residues in the oligomerization interface are largely hydrophobic, although some hydrophilic amino acids are present at the periphery of the interface. Within the oligomerization interface Trp136 is among the most extensively engaged residues, embedded deeply within a hydrophobic pocket on the adjacent protomer formed by Phe83, Ala110, Leu111, and Trp169. A hydrogen-bonding network centered on Arg119 also links adjacent protomers (Fig. 4b), as does a threefold symmetric aromatic stacking interaction among Phe191 residues from each protomer (Fig. 4c). Importantly, the oligomerization interface is highly conserved in sequence, attesting to its functional importance.

Size-exclusion experiments with multi-angle light scattering (SEC-MALS) showed that purified Sigma1 in detergent exists in a range of oligomeric states, with molecular weights ranging from at least 140 up to 400 kDa (Schmidt et al. 2016). Similarly, other biochemical and pharmacological work has shown that Sigma1 exists in a range of oligomeric states in detergents, with high-molecular-weight species being stabilized by ligands (Gromek et al. 2014). Cell-based fluorescence resonance energy transfer (FRET) studies have shown similar effects, as well as revealed agonist stabilization of low-molecular-weight species (Mishra et al. 2015). Taken together, these data indicate that oligomerization is an important feature of Sigma1 function, although the

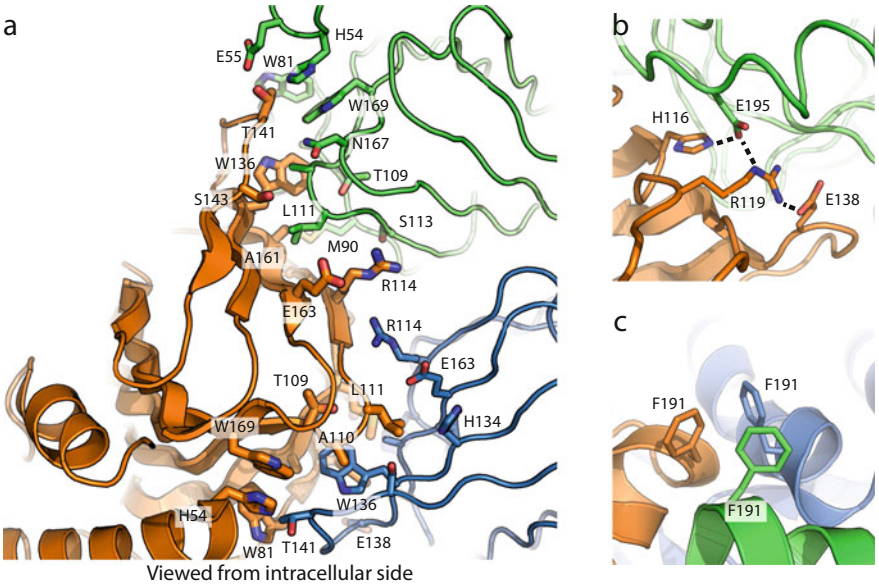

Fig. 4 Oligomerization: The interactions among Sigma1 protomers are extensive, involving dozens of amino acids primarily on the cytosolic face of the trimer. Viewed from the cytosolic face most of the oligomerization contact residues are resolved, shown as *sticks*

mechanistic details of oligomerization changes and any regulation thereof remain to be fully elucidated.

5 Implications for Sigma1 Function

While the Sigma1 receptor has been extensively studied by ligand-binding assays, the identity of the endogenous ligand, if any, remains unclear. Proposed ligands include dimethyltryptamine (Fontanilla et al. 2009) (DMT), but this has been called into question (Keiser et al. 2009) because of the much higher affinity of DMT at serotonergic receptors including the 5-HT2A receptor, which binds to DMT with 100-fold higher affinity than does Sigma1. Moreover, the behavioral effects of DMT administration are abrogated in 5-HT_{2A} receptor knockout mice (Keiser et al. 2009). Given these results, the identity of any endogenous Sigma1 ligand remains unclear.

Indeed, it is possible that the term "receptor" is something of a misnomer, and Sigma1 may function in an altogether different manner than conventional receptor families. One intriguing possibility is suggested by the close sequence homology between Sigma1 and the fungal sterol isomerase *ERG2*. While it has been demonstrated that Sigma1 cannot complement *ERG2* gene deletion in yeast (Hanner et al. 1996), it remains possible that Sigma1 possesses enzymatic activity that has yet to be discovered. Alternatively, Sigma1 may be the result of evolutionary repurposing

of an enzyme to a receptor, converting an active site into a ligand-binding site for regulation of receptor function.

In addition to pharmacological research, Sigma1 has been extensively probed in cell biological studies. This work has offered insight into a wide variety of aspects of Sigma1 activity, suggesting a possible role as a multifunctional regulator of transmembrane signaling. Interactions have been reported with GPCRs (Kim et al. 2010), ion channels (Aydar et al. 2002; Balasuriya et al. 2014), and chaperone proteins (Hayashi and Su 2007), among many others (Su et al. 2016). Current structural data are insufficient to comment substantively on the nature of such interactions, but this will doubtless be an important area for future research. Importantly, the advent of structural data now allows more rational construct design and analysis for cellular work. In particular, many previous experiments used constructs designed based on incorrect topological models that identified residues 100–223 as the carboxy-terminal domain. We now know that residues 33–99 are also integral parts of this domain, contributing two out of ten beta strands and two out of four alpha helices. Accordingly, the interpretation of research that used only partial fragments of this domain may need to be reconsidered.

6 Outlook

With the advent of high-resolution structural data for Sigma1, the field is poised for new insights and reconsideration of previous models. The discovery that Sigma1 possesses only a single transmembrane domain in particular highlights the risks associated with overreliance on a single model, and should now guide informed design of modified receptor constructs in future work. Despite the important insights offered by recent structures, other key questions remain unanswered and several areas for future research are highlighted below. In the long term, a complete understanding of Sigma1 biology will require a molecular understanding of ligand binding, efficacy, and regulation of interactions with other proteins. In each respect, structural biology is likely to play a pivotal role.

6.1 Relationship to Erg2p and Enzymatic Activity

The sequencing of Sigma1 20 years ago offered the first clear connection to a protein of well-described function, revealing sequence similarity to the yeast sterol isomerase Erg2p. In fungi, this enzyme plays an essential role in ergosterol biosynthesis, catalyzing the transfer of a double bond between the C8 and C9 positions to a new site between the C8 and C7 carbons. In humans, the analogous reaction is catalyzed by the emopamil-binding protein (EBP), which is unrelated to Sigma1 and Erg2p in primary sequence (Moebius et al. 1997). The sequence similarity between Sigma1 and Erg2p implies that the latter is likely to possess a similar membrane-embedded fold. This would allow the hydrophobic lipid substrate (fecosterol) to access the

catalytic site, while the equally hydrophobic product (episterol) can then escape directly into the bilayer.

Despite their sequence similarity and conservation of the catalytic/ligand-binding glutamate (Glu174 in yeast Erg2p, Glu172 in human Sigma1), the Sigma1 receptor fails to complement *ERG2* gene deletion in yeast (Hanner et al. 1996). This fact has been taken as evidence for a lack of Sigma1 catalytic activity. Nonetheless, Sigma1 has not been directly assessed for its ability to catalyze the analogous transformation in cholesterol, and it remains possible that an enzymatic activity like that of EBP may in fact be present. The development of robust procedures for purification of homogenous, functional Sigma1 should now allow straightforward assessment of this possibility in the near future.

6.2 Molecular Efficacy and Oligomerization

Many Sigma1 ligands have been classified as agonists and antagonists on the basis of their effects on animals (Nguyen et al. 2015). However, little is known regarding the molecular basis for ligand efficacy in terms of the specific receptor conformation(s) stabilized by agonists vs. antagonists. Cellular FRET data suggest a possible role for at least some Sigma1 antagonists in stabilizing high-molecular-weight oligomers, while certain agonists suppress oligomerization (Mishra et al. 2015). Nonetheless, the molecular mechanistic basis for these effects remains unknown. While it is increasingly apparent that oligomerization is a key aspect of Sigma1 function, its exact role and connection to ligand efficacy are likely to be important areas of research in years to come. In particular, elucidation of distinct conformations/oligomerization states is poised to be an important area for Sigma1 structural biology.

6.3 Interactions with Other Proteins

Sigma1 interactions with other proteins have been the subject of intense investigation for decades, with a wide range of proteins proposed as interaction partners (Su et al. 2016). Key areas for future work include validating these interactions with purified proteins, mapping sites of interaction, and determining the molecular basis for regulation of Sigma1/effector protein interactions. Recent advances in high-resolution electron microscopy (Liao et al. 2013) are particularly exciting, as these techniques may allow investigation of Sigma1 complexes with high-molecular-weight putative binding partners like inositol phosphate receptors, for which electron microscopy structural data has recently become available (Fan et al. 2015).

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3D Homology Model of Sigma1 Receptor

Erik Laurini, Domenico Marson, Maurizio Fermeglia, and Sabrina Pricl

Contents

Abstract

This chapter presents the three-dimensional (3D) model of the Sigma1 receptor protein as obtained from homology modeling techniques. We show the applicability of this structure to docking-based virtual screening and discuss combined in silico/in vitro mutagenesis studies performed to validate the structural features of the Sigma1 receptor model and to qualify/quantify the prominent role of specific amino acid residues in ligand binding. The validation of the virtual 3D Sigma1 receptor model and its reliable applicability to docking-based virtual

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screening is of significance for rational ligand design, even in light of the recently reported crystal structure for the Sigma1 receptor.

Keywords
Homology modeling • In silico mutagenesis • In vivo mutagenesis • MM/PBSA • Model validation

1 Introduction

The Sigma1 gene (7 kbp), consisting of four exons and four introns, is located on the human chromosome 9, band p13 (Prasad et al. 1998), a region associated with several psychiatric disorders (Ishiguro et al. 1998; Ohi et al. 2011). Its encoded protein, the Sigma1 receptor, was cloned from guinea pig liver for the first time in 1996 (Kekuda et al. 1996); shortly afterward, it was obtained from rat and mouse brain, rat kidney, and a human choriocarcinoma cell line (Hanner et al. 1996; Seth et al. 1998a, b; Pan et al. 1998; Mei and Pasternak 2001). The polypeptide consists of 223 amino acids, with a predicted molecular weight of 25.3 kilodaltons (kDa). Sigma1 receptors of different species share very high sequence identity (>93%) but do not show homology to any other mammalian protein. Intriguingly, the Sigma1 receptor reveals a 30% identity, a 67% homology, and a similar ligand binding profile to the yeast Δ^8/Δ^7-sterol isomerase, encoded by the *erg2* gene (Moebius et al. 1997; Hanner et al. 1996; Chen et al. 2007). Yet, the Sigma1 receptor is devoid of sterol isomerase activity.

To date, no endogenous ligand of the Sigma1 receptor has been unequivocally identified. However, neurosteroids such as progesterone and pregnenolone have been discussed as potential natural Sigma1 receptor ligands (Su et al. 1988; Ganapathy et al. 1999). Sigma1 receptors bind cholesterol and accumulate in cholesterol-dense regions of the cell (e.g., lipid rafts) (Palmer et al. 2007). Recently, *N*,*N*-dimethyltryptamine was also postulated to be the endogenous Sigma1 receptor ligand (Fontanilla et al. 2009), despite its weak inhibition constant ($K_i = 15.4$ μM). In contrast, several synthetic molecules belonging to different structural classes have been reported to bind the Sigma1 receptor with very high affinity and selectivity. Dextrorotatory benzomorphans like (+)-pentazocine and (+)-*N*-allylnormetazocine (a.k.a. (+) SKF-10047), which have been classified as Sigma1 receptor agonists, bind the receptor in the low nanomolar range (e.g., $K_i = 3$ nM and 18 nM, respectively) (McCann and Su 1990; Tam and Cook 1984; Bowen et al. 1993; Cagnotto et al. 1994; Klouz et al. 2002). Other ligands such as haloperidol or 4-methoxy-3-*N*,*N*-dipropylbenzeneethanamine (NE-100), classified as Sigma1 antagonists (Tam and Cook 1984; McCann and Su 1990; Okuyama and Nakazato 1996), also show high binding affinity to the Sigma1 receptor ($K_i = 4$ nM and 13 nM, respectively). Psychostimulants (e.g., cocaine, $K_i = 2$ μM (Sharkey et al. 1988)), fungicides (e.g., fenpropimorph, $K_i = 0.005$ nM (Moebius et al. 1997)), anxiolytics (e.g., opipramol, $K_i = 50$ nM (Rao et al. 1990), anti-Alzheimer drugs (e.g., donepezil, $K_i = 14.6$ nM (Koki et al. 1999), centrally active antitussives (e.g., (+)-dextromethorphan, $K_i = 151$ nM (Chou et al. 1999)), and antidepressants (e.g.,

fluvoxamine, sertraline, and *S*-(+) fluoxetine, K_i = 36 nM, 57 nM, and 120 nM, respectively (Narita et al. 1996)) are also endowed with high to moderate affinity to the Sigma1 receptor.

Further investigations of the *SIGMAR1* gene (Ganapathy et al. 1999) revealed the existence of a splice variant at exon 3. The resulting Sigma1 receptor variant, bearing a 31-residue deletion mutation (from Arg119 to Gly149), was no longer able to bind ligands like [^{3}H]haloperidol, [^{3}H](+)-pentazocine, and [^{3}H]-(+)-3-(3-hydroxyphenyl)-1-propylpiperidine ([^{3}H](+)-PPP). The same group analyzed a Sigma1 receptor variant bearing three point mutations (Ala13Thr, Leu28Pro, and Ala86Val), and found that the binding affinity of [^{3}H]haloperidol for this mutated receptor isoform was reduced by 60% compared to the wild type protein.

Initial studies of the structure of the Sigma1 receptor, based on hydrophobicity analysis of receptor primary structure, led to the hypothesis that the protein was characterized by one single transmembrane domain, spanning residues Ala92-Gly112 as shown in the left panel of Fig. 1 (Kekuda et al. 1996; Seth et al. 1998a, b). Subsequent reports proposed that the receptor comprises two transmembrane domains (shown in the right panel of Fig. 1), namely the TMI spanning residues Ala10 to Leu30, and the TMII spanning residues Gln80 to Leu100 (Pan et al. 1998; Aydar et al. 2002). In addition, a third putative membrane flanking hydrophobic region spanning residues Gly176 to Thr203 was proposed (Palmer et al. 2007; Hayashi and Su 2007).

Finally, the same hydrophobicity analysis revealed two more hydrophobic domains within the primary sequence of the Sigma1 receptor: the so-called *steroid binding domain like* I and II (SBDL I and II), thus named because of their homology with the *steroid binding domains* of yeast and other fungal Δ^8/Δ^7-sterol isomerases (Chen et al. 2007).

SBDL I and II comprise residues Gly91 to Thr109 (SBDL I) and Gly176 to Gln194 (SBDL II), respectively. SBDL I partially overlaps with the TMII domain,

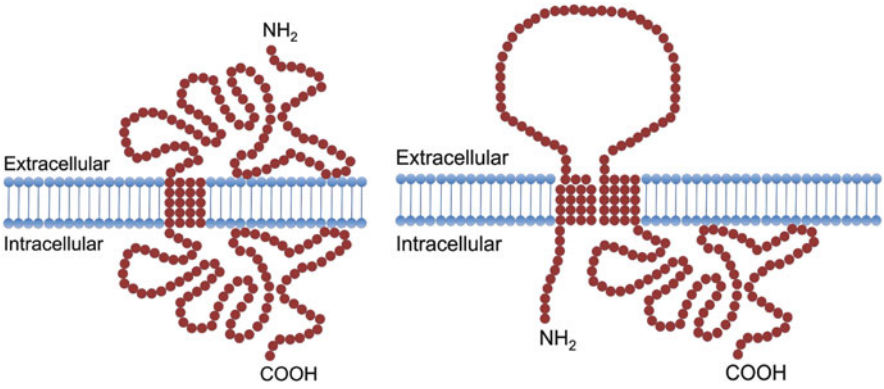

Fig. 1 (*Left*) First cartoon model of the Sigma1 receptor with only one transmembrane domain. (*Right*) Cartoon model of the Sigma1 receptor with two transmembrane domains

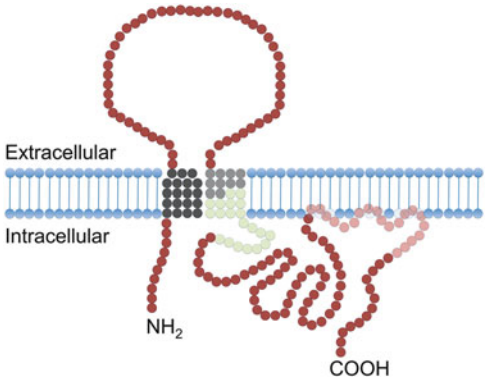

Fig. 2 Cartoon model of the Sigma1 receptor with two transmembrane domains and a third membrane flanking region. Residues belonging to the TMI and TMII regions are as *dark* and *light gray dots*, respectively. Amino acids of the SBDL I and SBDL II domains are shown as *light green light pink dots*, respectively

whereas SBDL II is positioned in the membrane flanking region of the receptor C-terminal end, as shown in Fig. 2.

Two other structurally important elements have been identified. The first is a typical arginine-arginine endoplasmic reticulum retention signal near the N terminus of the protein, and the second, two GlyXXXGly motifs, a signature that occurs with high frequency in membrane proteins that favor helix–helix interactions (Polgar et al. 2004).

In the last two decades site directed mutagenesis (Seth et al. 2001) and photoaffinity labeling experiments (Kahoun and Ruoho 1992) were performed to characterize the structure of the receptor and its binding site. According to these studies, the anionic amino acids Asp126 and Glu172 were found to be essential for haloperidol binding, most likely by interacting with a cationic moiety of the ligand. Replacement of residues Ser99, Tyr103, Leu105, and Leu106 – all belonging to the SBDLI – by alanine also revealed that a Tyr103Ala mutation had the strongest influence on binding of both agonist ((+)-pentazocine) and antagonist (NE-100) compounds. In separate studies, the tyrosine residues at positions 173, 205, and 206 were also found to be important for cholesterol binding (Yamamoto et al. 1999; Palmer et al. 2007; Pal et al. 2008). A photo-activated cocaine derivative bound to Asp188, however, ligand binding was abolished when more than 15 amino acids of the C-terminus of the receptor were removed (Kahoun and Ruoho 1992). Intramolecular transfer of the photoaffinity label [^{125}I]IABM indicated that the SBDLI and SBDLII of the Sigma1 receptor are juxtaposed. It was suggested that the receptor TMI, the SBDLI, and the SBDLII form the Sigma1 ligand binding site, since another photoaffinity label ([^{125}I]IAF) was detected both on the SBDLI and the SBDLII after enzymatic digestion (Pal et al. 2008). Altogether, these findings led to the hypothesis that the Sigma1 ligand binding site is formed by SBDL I, SBDL II, and a part of the TMI domain, as schematically illustrated in Fig. 3.

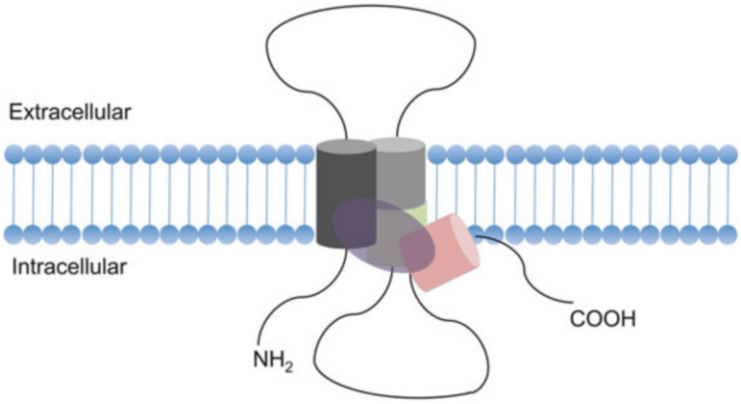

Fig. 3 Cartoon of the putative Sigma1 structure and of its ligand binding site consisting of TMI (*dark gray*), SBDL I (*light green*), and SBDL II (*light pink*) as hypothesized from photoaffinity studies. The binding site is *encircled* in the transparent, *light purple area*

2 Development of the Three-Dimensional Model of the Sigma1 Receptor

In 2011 our group developed for the first time a 3D model for the Sigma1 receptor (Laurini et al. 2011) based on a combination of molecular modeling and simulation methodologies, involving the following sequential steps: (a) generation and optimization of the complete 3D model of the receptor by homology modeling techniques (Bordoli et al. 2008); (b) 3D homology model refinement exploiting information derived from ligand docking (Evers et al. 2003) and preliminary mutagenesis experiments (Seth et al. 2001) as spatial restraints; (c) docking of a series of bioactive ligands including, among others, (+)-pentazocine and haloperidol, into the putative binding site, estimation of their binding affinity via the Molecular Mechanics/Poisson-Boltzmann Surface Area (MM/PBSA) method (Srinivasan et al. 1998), and comparison of the in silico ligand scoring with available experimental activities (Zampieri et al. 2009); and (d) design of a set of new ligands with different degrees of affinity for the protein based on the derived receptor model to test the predictive capability of the Sigma1 receptor 3D model.

The computational protocol adopted in steps (a) and (b) involved modeling the three receptor TM regions (i.e., the three α-helices spanning residues 10–30, 80–100, and 180–200, respectively), and prediction of the overall secondary structure of the protein, which included a few β-strands in the C-terminal half (residues 111–116, 133–135, 144–146, and 158–164), and some loops, as shown in the left panel of Fig. 4.

For this approach, four different protein sequences having identity $\geq$30% with specific portions of the Sigma1 receptor sequence and available X-ray structure deposited in the Protein Data Bank (i.e., 3CIA.pdb, 1I24.pdb, 2Z2Z.pdb, and 2Q8I.

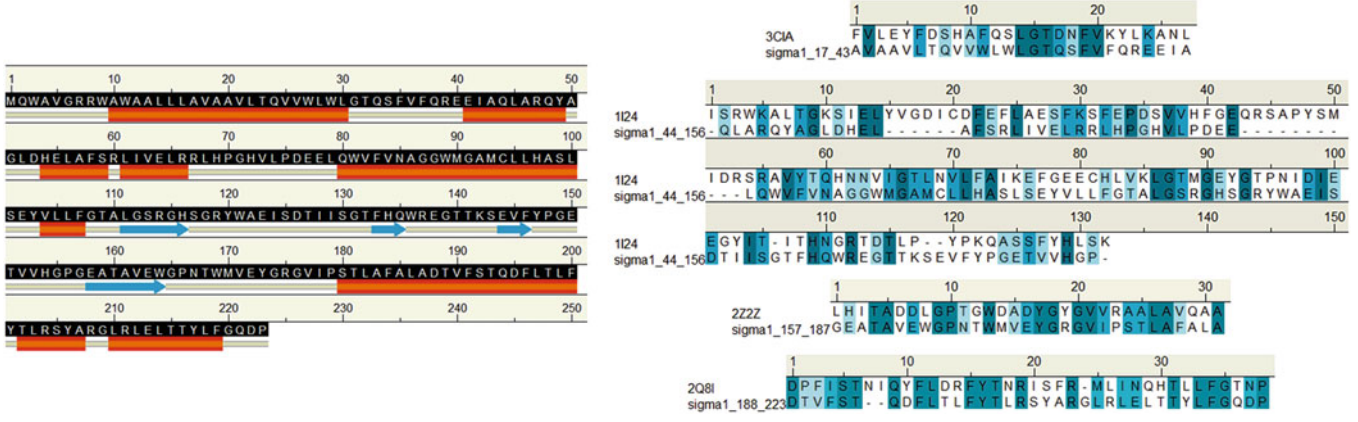

Fig. 4 (*Left*) General secondary structure prediction of the Sigma1 receptor. The main protein structural motifs (a-helices, b-sheets, and loop/coils) are highlighted below the primary sequence as follows: *orange/red stripes*: α-helices; *light blue arrows*: β-sheets; *gray stripes*: loops/coils. (*Right*) Sequence alignment of different portions of the human Sigma1 receptor with (from top to bottom): cold-aminopeptidase (3CIA.pdb), Udp-sulfoquinovose synthase (1I24.pdb), Tk-subtilisin (2Z2Z.pdb), and pyruvate dehydrogenase kinase isoform 3 (2Q8I.pdb) (Laurini et al. 2011)

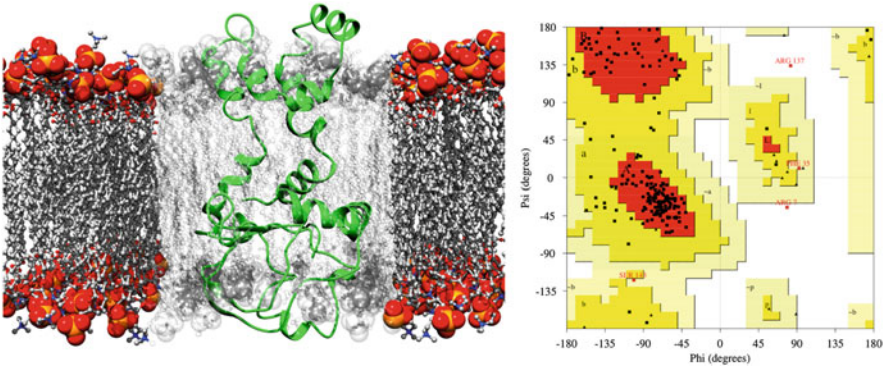

Fig. 5 (*Left*) Side view of the equilibriums structure of the Sigma1 receptor 3D homology model in a solvated membrane environment. The protein secondary structure is depicted as a *green ribbon*. POPC molecules are shown in mixed representation of CPK *spheres* and *balls*-and-*sticks*. Atom color code: O, *red*; C, *light gray*; N, *light blue*; and P, *orange*. The portion of the lipid bilayer surrounding the Sigma1 receptors has been rendered transparent for graphical purposes. Hydrogen atoms, water molecules, ions and counterions are not shown to maintain clarity. (*Right*) Ramachandran plot generated from the MD refined 3D homology model of the Sigma1 receptor (Laurini et al. 2011)

pdb) were retrieved. Accordingly, each corresponding receptor/template sequence was aligned (Fig. 4, right panel), and the Cartesian coordinates for the Sigma1 residues in structurally conserved regions were obtained from the corresponding sequence in the template PDB file. The first part of the N-terminal domain of the protein, spanning residues 1–16, showed no homology with any other proteins in all queried databases and thus was built de novo. Finally, the overall receptor 3D structure was built by linking the different template-based homology models and creating and optimizing the missing loop portions via several refinement processes. The structure was slightly adjusted to fully match the results of secondary structure predictions, and optimized for backbone and side chain conformation.

This initial 3D model was inserted manually into a 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine (POPC) membrane model, with the two transmembrane helices parallel to the *xy* plane, and with *z* in the direction normal to the membrane. Next, the entire system was subjected to extensive molecular dynamics (MD) refinement in water at the physiological ionic strength of 150 mM NaCl to attain the energetically stable and favorable protein conformation illustrated in the left panel of Fig. 5.

The MD-refined homology model of the Sigma1 receptor obtained at the end of the optimization procedure had high stereochemical quality, with 221 over 223 total receptor residues found in the most favored regions, while only two residues residing in generously allowed regions of the corresponding Ramachandran plot (Ramachandran et al. 1963) (Fig. 5, right panel). Other stereochemical parameters such as dihedral angles, covalent geometry, and planarity were also examined. Thus, PROCHECK G-factors (Laskowski et al. 1993) were all close to zero, and all

values were well within the acceptable limits.[1] The overall packing quality of the 3D Sigma1 receptor optimized model was inspected using the atomic contact analysis of WHATIF (Vriend 1990), which yielded a z-score of −0.89.[2] The packing quality of each single receptor amino acid was also evaluated by running VERIFY-3D (Lüthy et al. 1992): the compatibility score above zero in the corresponding graph (an indication of the residue side-chain environment) suggested that the model is characterized by an overall self-consistency in terms of sequence-structure compatibility. The excellent quality of the overall 3D homology model of the Sigma1 receptor was also reflected in the value of the corresponding PROSA normalized z-score value (Sippl 1993) of 0.98.[3] Such a high value of the PROSA z-score approaches those typical of high resolution crystal structures, further supporting that the proposed model is characterized by very good backbone conformation quality.

It is noteworthy that the overall accuracy of a protein homology model is related to the percentage sequence identity (SI) upon which the model is based, together with the relationship between the structural and sequence similarity of target and template proteins. High-accuracy comparative models are generally based on more than 50% SI to their templates. Medium-accuracy comparative models are based on 30–50% SI, while low-accuracy comparative models rely on less than 30% of SI. Other factors such as template selection and alignment accuracy usually have a significant impact on the quality of the resulting final model, especially for models based on less than 40% sequence identity to the templates (Baker and Sali 2001). Specifically, many methods often fail to correctly align protein pairs with 20–30% pairwise sequence identity, and indeed a portion of the Sigma1 receptor was modeled in this so-called twilight zone (Rost 1999). However, given that (1) it is also often possible to correctly predict features of the target protein that do not occur in the template structure, (2) errors in functionally important regions in homology models are relatively low because the functional regions (e.g., binding sites) tend to be more conserved in evolution than the rest of the fold, and (3) all Sigma1 receptor ligands considered for successive docking and affinity scoring (vide infra) against the receptor 3D homology model yielded K_i values in line with the corresponding experimental data, it can be confidently concluded that the proposed homology model is characterized by overall correct protein folding.

The Sigma1 receptor homology 3D model and the relevant binding site were identified by exploiting the currently available preliminary information on sequence–structure relationships and mutagenesis studies (Seth et al. 2001) and some ligand-binding pharmacophore requirements (Zampieri et al. 2009). To validate the 3D model, a set of nine Sigma1 ligands were docked into the protein binding cavity and subsequently scored for receptor affinity via MM/PBSA calculations (step c) (Laurini et al. 2011). Haloperidol, (+)-pentazocine, and

[1]Reasonable values for the G-factor in PROCHECK fall between 0 and −0.5, the best models displaying values closest to 0.

[2]WHATIF normality index (z-score) values for valid structures should be greater than −5.0.

[3]PROSA normalized z-score values >0.70 are indicative of a good structure.

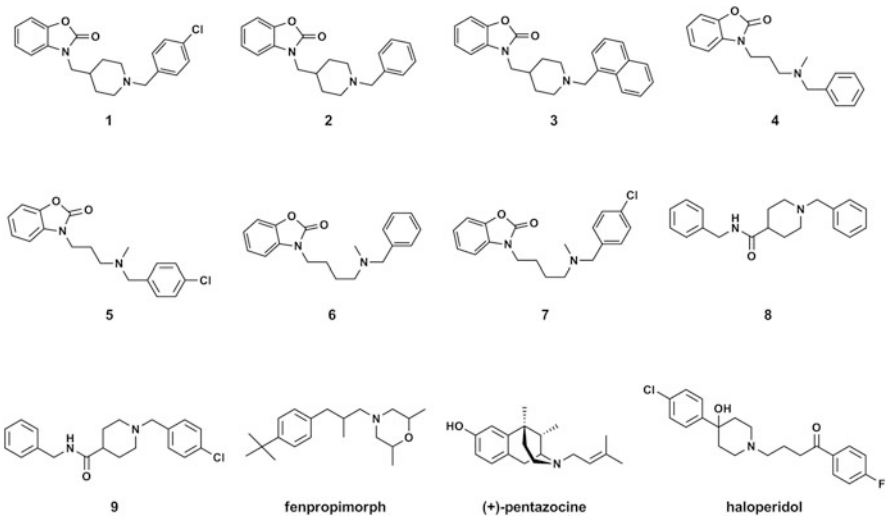

Fig. 6 Chemical structure of the set of nine ligands, fenpropimorph, (+)-pentazocine, and haloperidol used for docking and affinity scoring against the Sigma1 receptor 3D homology model (Laurini et al. 2011)

fenpropimorph – three structurally diverse Sigma1 receptor ligands – were included for further testing and comparison (Fig. 6).

According to the MM/PBSA theory, the free energy of binding ΔG_{bind} between the Sigma1 receptor and each of its ligands can then be calculated as:

$$\Delta G_{\text{bind}} = \Delta E_{\text{MM}} + \Delta G_{\text{solv}} - T\Delta S \tag{1}$$

where ΔG_{bind} is the ligand binding free energy in water, ΔE_{MM} is the ligand/receptor interaction energy, ΔG_{solv} is the solvation free energy, and $-T\Delta S$ is the conformational entropy contribution to binding.

Equation (1), ΔE_{MM} is calculated from molecular mechanics (MM) interaction energies as: $\Delta E_{\text{MM}} = \Delta E_{\text{vdW}} + \Delta E_{\text{ele}}$, where ΔE_{vdW} and ΔE_{ele} are the van der Waals and Coulombic contribution to ligand/protein association. ΔG_{solv}, that is the free energy exchange in ligand and protein desolvation upon complex formation, is also given by the sum of two terms, the electrostatic contribution to solvation ΔG_{PB} (which can be obtained by numerically solving the Poisson-Boltzmann equations and calculating the relevant electrostatic energy according to the electrostatic potential) (Gilson et al. 1988) and the nonpolar ΔG_{NP} contribution to solvation, proportional to the so-called solvent accessible surface (Sitkoff et al. 1994). The last term in Eq. (1), i.e., the change in solute entropy upon association $-T\Delta S$, can be calculated through normal-mode analysis (Wilson et al. 1995).

Once the values of the ΔG_{bind} are estimated, the corresponding K_i values for each ligand/protein ensemble can be obtained using the fundamental thermodynamic relationship:

$$\Delta G_{\text{bind}} = RT \ \ln \ K_i \tag{2}$$

The application of the MM/PBSA to scoring the binding affinity of the selected ligand set to the Sigma1 receptor not only correctly ranked the entire series of nine compounds but also the three structurally diverse prototypical Sigma1 ligands haloperidol, (+)-pentazocine, and fenpropimorph. Indeed, for all of these molecules, an excellent agreement ($R^2 = 0.93$) between computed and experimental affinities of these ligand series was obtained (see Fig. 7, left panel). This represented the first validation of the 3D homology model of the Sigma1 receptor and its ligand binding site.

The set of compounds used in step (c) included a series of benzamide and benzoxazolone derivatives, characterized by K_i values spanning 4 orders of magnitude (0.098–1,147 nM), in order to test the ability of the model to rank high-, intermediate-, and low-affinity ligands. Next, the high-affinity benzoxazolone derivative **1** (Fig. 6, $K_i = 0.098$ nM) was specifically analyzed in silico to characterize all important protein–ligand interactions in the receptor binding site (right panel in Fig. 7). According to these simulations, the anionic amino acid Asp126

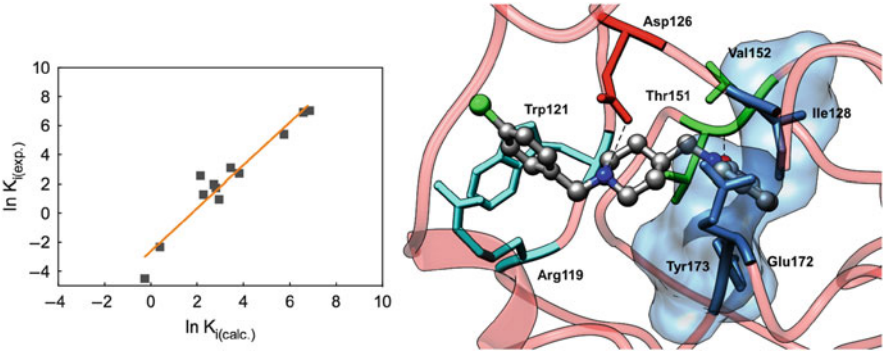

Fig. 7 (*Left*) Linear correlation obtained between the calculated Sigma1 receptor ligand K_i values and the corresponding experimental K_i values (correlation coefficient $R^2 = 0.93$) for the 12 compounds shown in Fig. 5. (*Right*) Zoomed view of the high-affinity benzoxazolone derivative **1** ($K_i = 0.098$ nM) in complex with the Sigma1 receptor 3D homology model. The compound is shown as *atom-colored sticks*-and-*balls* (C, *gray*; O, *red*; N, *blue*; Cl, *green*; H atoms are not shown for clarity). The receptor is portrayed as a *salmon pink ribbon*. The main protein residues involved in ligand binding are visualized as *sticks*, *labeled*, and *colored* according to the underlying interaction with the receptor: Arg119 and Trp121, π interactions, *cyan*; Asp126, salt bridge (SB), *red*; Ile128, Glu172, and Tyr173, hydrophobic interactions, *steel blue*; Thr151 and Val 152, hydrogen bond (HB), *green*. The receptor hydrophobic cavity lined by the side chains of Ile128, Glu172, and Tyr173 is highlighted by the relevant van der Waals surface (*steel blue*). SB and HB are evidenced by *black broken lines*. Water and membrane molecules, ions and counterions are not shown for clarity (Laurini et al. 2011)

forms a salt bridge with the basic amino moiety in the piperidine ring, whereas Glu172 interacts with the benzoxazolone heterocycle by electrostatic interactions. The hydrogen bond formed between the NH of the protein backbone peptidic bond between Thr151 and Val152 and the oxygen atom of the carbonyl moiety of the benzoxazolone anchors the compound in place. π–π stabilizing interactions between Trp121 and the *p*-chlorophenyl ring as well as between Tyr173 and the phenyl ring of the benzoxazolone part of the ligand further stabilize the ligand–protein complex. The basic residue Arg119 interacts with the *p*-chlorophenyl moiety of the compound by van der Waals forces, while Ile128 stabilizes the molecule in its binding pocket by hydrophobic interactions with the aromatic phenyl ring of the benzoxazolone system.

The last step of preliminary validation of the 3D Sigma1 receptor model and of its binding site (step d) involved the design of a new group of ligands using the spatial and energetic information derived from the 3D model itself. Using this approach, compounds **10**, **11**, and **12** were conceived (Fig. 8) and ranked as high, moderate, and low affinity ligands in the Sigma1 receptor 3D model, according to the in silico $\Delta G_{bind}/K_i$ values shown in Table 1.

Importantly, these predicted affinities were confirmed by the corresponding experimental values of binding constant K_i, thereby validating the predictive features of the Sigma1 receptor 3D model.

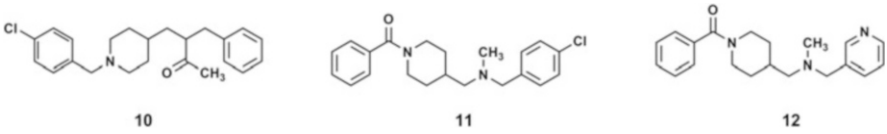

Fig. 8 Chemical structures of the new ligands design to test the predictive validity of the Sigma1 receptor 3D homology model in assisted drug design/virtual screening

Table 1 Binding free energies ΔG_{bind} for the set of three new Sigma1 receptor ligands designed exploiting the developed Sigma1 receptor 3D homology model

	10	11	12
ΔG_{bind} (kcal/mol)	-11.31 ± 0.38	-10.02 ± 0.35	-7.75 ± 0.37
K_i (calculated) (nM)	5.20	45.7	2,100
K_i (experimental) (nM)	1.85 ± 0.25	30.3 ± 1.69	$1{,}578 \pm 135$

The experimental and calculated K_i, as estimated from the corresponding ΔG_{bind} values, are also reported for comparison. Calculated K_i values were obtained from the corresponding ΔG_{bind} using Eq. (2) (Laurini et al. 2011)

3 Validation of the Three-Dimensional Model of the Sigma1 Receptor

3.1 Validation by Ligand Binding

Since its original presentation, the 3D model of the Sigma1 receptor has been the subject of extensive validation by ligand binding performed by our group in collaboration with other teams (Laurini et al. 2012, 2013, 2014; Meyer et al. 2012; Rossi et al. 2013a, b; Zampieri et al. 2014, 2015; Weber et al. 2014). For example, in the first paper of the series (Laurini et al. 2012), the 3D model of the Sigma1 receptor was used to design 33 new structurally diverse ligands, based on phenyl(piperidin-1-yl)methanone, *N*-benzylpiperidin-4-yl-acetamide, and *N*-benzylpiperidin-4-yl-benzamide scaffolds, respectively. Their rank order potency in Sigma1 receptor binding was calculated by molecular dynamics simulations. Also, the main interactions involved in receptor/ligand binding were analyzed by applying a *per residue* free energy deconvolution (Gohlke et al. 2003) and in silico alanine scanning mutagenesis (Massova and Kollman 1999). Subsequently, all compounds were synthesized and tested for empirical Sigma1 binding affinity in vitro. The agreement between in silico and in vitro results (Fig. 9, left panel) confirmed the reliability of the proposed Sigma1 3D model in the a priori prediction of the affinity of new Sigma1 ligands. Moreover, it also corroborated the currently available biochemical data concerning the Sigma1 receptor amino acid residues considered essential for ligand binding (Fig. 9, right panel).

In a second example, the work of Meyer et al. (2012) marked another milestone in deciphering structural details of ligand binding to the Sigma1 receptor. In this

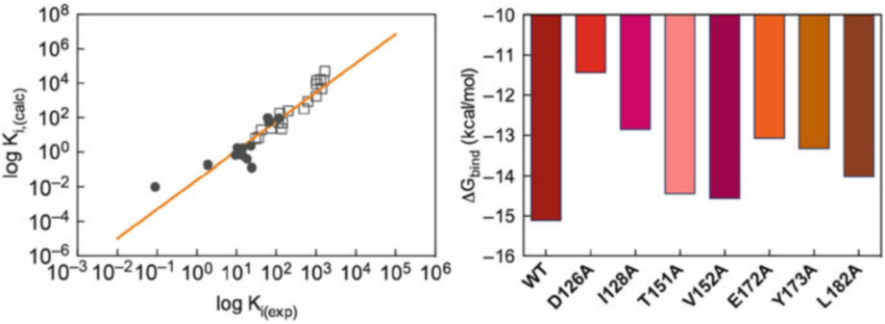

Fig. 9 (*Left*) Comparison of experimental and Sigma1 3D homology model-based predicted K_i values for the 33 compounds featuring the phenyl(piperidin-1-yl)methanone (*open square*) and the *N*-benzylpiperidin-4-yl-acetamide and *N*-benzylpiperidin-4-yl-benzamide scaffolds (*filled circles*), respectively ($R^2 = 0.89$). (*Right*) Free energy of binding (ΔG_{bind}) values of WT and mutant Sigma1 3D receptor models bearing an alanine residue at positions 126, 128, 151, 152, 172, 173, and 182 in complex with one *N*-benzylpiperidin-4-yl-acetamide derivative. As can be seen, the compound is less affine to all mutant receptor isoforms (i.e., the corresponding ΔG_{bind} values are less negative with respect to the wild-type (WT) protein), indicating the importance of the selected residue – and of Asp126 in particular – in ligand binding (Laurini et al. 2012)

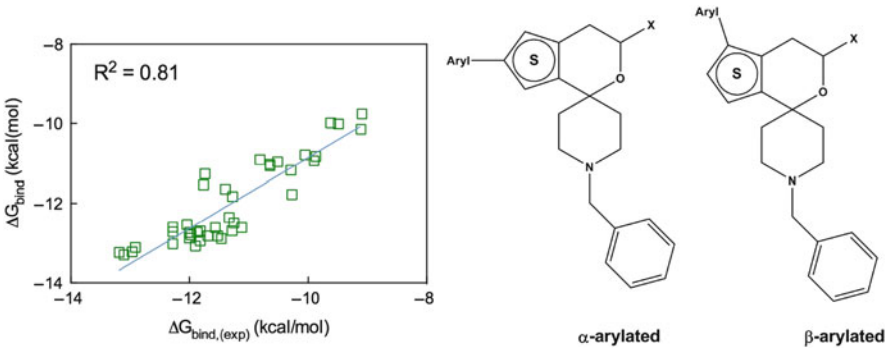

Fig. 10 Correlation (*left panel*) between experimental and in silico predicted free energies of binding (and, hence K_i values, see Eq. (2)) values obtained by docking-MM/PBSA scoring 40 regioisomeric spirocyclic thiophene derivatives bearing an aryl moiety on the α-position or β-position (*right panel*) using the 3D Sigma1 receptor model (Meyer et al. 2012)

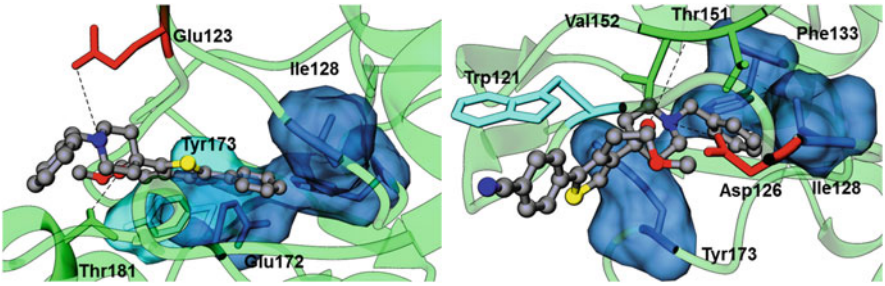

Fig. 11 Equilibrated MD snapshots of template compounds of α-arylated (*left*) and β-arylated (*right*) derivatives in complex with the Sigma1 receptor. The images are zoomed views of the receptor binding site. The protein structure is depicted as a transparent *lime green* and *forest green ribbon*, respectively, while both ligands are shown in *atom-colored sticks* and *balls* (C, *gray*; O, *red*; S, *yellow*; N, *blue*). Hydrogen atoms, water and membrane molecules, ions and counterions are omitted for clarity. The amino acid residues mainly involved in the interaction with the ligands are highlighted as *colored sticks* and *labeled*. Salt bridge and H-bond interactions are shown as *dotted black lines* (Meyer et al. 2012)

study, an excellent correlation (Fig. 10, left panel) was again obtained between experimental and in silico K_i values estimated by docking and MM/PBSA scoring 40 regioisomeric spirocyclic thiophene derivatives bearing an aryl moiety on the α-position or β-position (Fig. 10, right panel) using the 3D model of the Sigma1 receptor. Most importantly, however, these calculations further unveiled a reverse binding mode of the molecules bearing the phenyl substituent in the β position with respect to those featuring the same group in the α position, as shown in Fig. 11; such an opposite molecular orientation was required in order for these classes of compounds to fulfill all receptor/ligand stabilizing interactions (see Fig. 11).

3.2 Validation by In Silico/In Vitro Mutagenesis

In a recent study, we confirmed previously reported structural features of the Sigma1 receptor and its binding site, and we further characterized the role of several key residues involved in receptor–ligand binding (Brune et al. 2014). Specifically, we performed a combined in silico/in vitro study to analyze the molecular interactions of the Sigma1 receptor with its prototypical agonist, (+)-pentazocine. Accordingly, first 23 alanine (or glutamic/aspartic acid)-mutant Sigma1 receptor isoforms were generated, and their interactions with (+)-pentazocine were determined experimentally (Fig. 12).

Next, the same mutagenesis scheme was reproduced in silico on the 3D homology model of the receptor, and all direct and/or indirect effects exerted by the mutant residues on the protein–agonist interactions were reproduced and rationalized, thereby casting a new light on the structural biology of the Sigma1 receptor and its ligand binding site.

The main results from this study highlighted the following structural features of the Sigma1 receptor.

Sigma1 Receptor Binding Site Residues The following interactions were found to be essential for (+)-pentazocine binding: (1) a permanent salt bridge between the NH^+ moiety of (+)-pentazocine and the COO^- group of Asp126 (with an average dynamic length (ADL) of 3.93 ± 0.09 Å), (2) a stable hydrogen bond between the carboxylate group of Glu172 and the hydroxyl substituent of (+)-pentazocine (ADL of 1.98 ± 0.04 Å), (3) a T-stacking π–π interaction between the side chains of

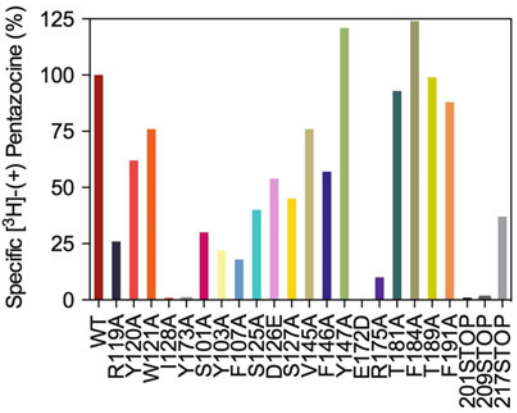

Fig. 12 Specific binding of [^{3}H](+)-pentazocine at a concentration of 40 nM to different alanine/glutamic/aspartic acid mutants to the human Sigma1 receptor. Specific binding refers to agonist binding to the wild-type (WT) receptor (100%, first column). A specific binding <30% indicates a strong influence of that particular amino acid on ligand binding, 30% ≤ specific binding ≤70% indicates a moderate influence on ligand binding, whereas a specific binding higher than >70% indicates no influence of the mutated residue on ligand binding (Brune et al. 2014). For readability, the single letter amino acid notation is adopted in this figure

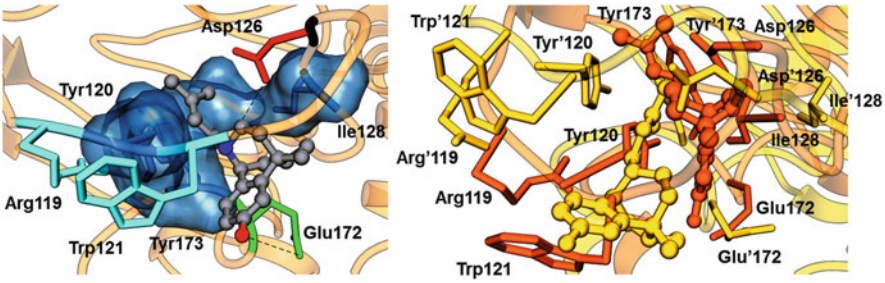

Fig. 13 (*Left*) Equilibrated molecular dynamics (MD) snapshot of the WT Sigma1 receptor in complex with (+)-pentazocine. The image is a close-up of the receptor binding site. The ligand is shown as *colored balls* and *sticks* (C, *gray*; N, *blue*; O, *red*). The protein residues mainly involved in the interaction with (+)-pentazocine are highlighted as *labeled colored sticks*. Salt bridges and H-bond interactions are shown as *solid* and *dotted black lines*, respectively. (*Right*) Comparison of the equilibrated MD snapshots of the WT Sigma1 receptor (*orange*) and the Tyr103Ala (*gold*) mutants in complex with (+)-pentazocine. In both panels, hydrogen atoms, water and membrane molecules, ions and counterions are omitted for clarity

Tyr120 and Trp121 and the heteroaromatic condensed rings of (+)-pentazocine, and (4) highly stabilizing van der Waals and electrostatic interactions between Arg119, Ile128, and Tyr173 and the aliphatic/aromatic portions of the ligand (see Fig. 13, left panel). The salt bridge and the hydrogen bond involving Asp126 and Glu172 are responsible for stabilizing contributions of −2.89 and −1.83 kcal/mol, respectively. Substantial van der Waals and electrostatic interactions are further contributed by Arg119 (−0.98 kcal/mol), Tyr120 (−1.49 kcal/mol), Trp121 (−1.37 kcal/mol), Ile128 (−2.04 kcal/mol), and Tyr173 (−1.95 kcal/mol). Thr151 and Val152 additionally contribute −0.36 and −0.38 kcal/mol, respectively, to the stabilization of (+)-pentazocine–Sigma1 receptor binding.

Mutating residues Arg119, Ile128, and Tyr173 within the Sigma1 receptor binding site into alanine lead to an almost complete loss of ligand binding, as predicted from simulation. Accordingly, the favorable hydrophobic interactions exerted by these two residues in the binding of (+)-pentazocine are lost upon alanine substitution, resulting in 2.47 kcal/mol and 2.70 kcal/mol decrease in affinity (ΔG_{bind}) for Ile128Ala and Tyr173Ala, respectively (in agreement with the experimental specific binding = 1% and 0.4% for the two residues, Fig. 12). On the other hand, the affinity of the Tyr120Ala and Trp121Ala Sigma1 receptor mutants for (+)-pentazocine was only partly reduced (specific binding values of 62 and 76%, respectively, Fig. 12). The relevant modeling analysis reveals that, although each of these two aromatic residues concurs in stabilizing (+)-pentazocine binding mainly via π–π interactions, the effect of mutating either of these two positions to alanine results in an apt rearrangement of the alternative residue side chain within the binding site and, hence, partially compensating receptor affinity loss, with an unfavorable variation in ΔG_{bind} of 0.68 kcal/mol for Tyr120Ala and 1.54 kcal/mol for Trp121Ala, respectively.

The position of residues Asp126 and Glu172, each carrying a negative charge each, within the Sigma1 receptor binding site is strategic for (+)-pentazocine binding. In this respect, the elongation of the residue side chain from aspartate to glutamate at position 126 is somewhat tolerated (affinity loss of 0.89 kcal/mol), resulting in a mild rearrangement of the protein binding pocket that preserves the main network of interactions between the ligand and the protein, the corresponding reduction in chain (specific binding 54%, Fig. 12). On the contrary, shortening the residue side chain in the Glu172Asp mutant fully abrogates the ability of the protein to bind the agonist, with a decrease of (+)-pentazocine/receptor affinity of 2.14 kcal/mol (specific binding of 0%).

Sigma1 Receptor Residues Belonging to the SBDL1 and SBDLII Domains A systematic substitution of the polar (Ser and Thr) and aromatic (Phe and Tyr) amino acids of these protein regions with alanine unequivocally demonstrated that while polar residues were required in the SBDLI domain to maintain the Sigma1 receptor binding site geometry, analogous substitutions in the SBDLII domain barely influenced (+)-pentazocine binding, if at all. Thus, mutating the polar and aromatic amino acids of SBDLI (Ser101, Tyr103, and Phe107) to alanine both in silico and in vitro led to a considerable decrease in the level of (+)-pentazocine binding (ΔG_{bind} loss in the range 2.13–1.86 kcal/mol, experimental specific binding between 18 and 30%, Fig. 12). A likely explanation of these results is that, according to the 3D Sigma1 model, all three alanine-mutated residues are in the proximity of its transmembrane domain and, during the long molecular dynamics simulation, they promote a substantial modification of both the binding site and the surrounding protein regions (see Fig. 13, right panel). As anticipated above, in the case of the SBDLII domain residues (Thr181, Phe184, Thr189, and Phe189), the presence of alanine at these positions only modest or nil variation in receptor affinity was predicted (ΔG_{bind} variation in the range 0.59–0.03 kcal/mol), consistent with the corresponding experimental findings (specific binding 93–100%, Fig. 12). A comparison of the membrane-bound 3D model of the wild-type (WT) and SDBLII mutated Sigma1 receptor isoforms reveals that the presence of mutations at the SBDLII domain does not lead to substantial alteration of the membrane and the protein binding site and/or overall structure. Accordingly, all ligand−receptor interactions detected for the WT complex are maintained in this as well as in all other SBDLII mutated complexes examined, with no subsequent significant differences in ligand binding mode or strength.

Of note, in a previous study SBDLII was postulated to be part of the Sigma1 receptor ligand binding site (Pal et al. 2008). Our studies do not support this hypothesis. It has been further proposed that SBDLII is responsible for anchoring the Sigma1 receptor to the membrane and, in so doing, stabilizing the 3D structure of the protein. Once again, our combined in vitro/in silico experiments do not support this model. Taken together, our findings lead to the conclusion that the SBDLI domain is part of the binding site of the Sigma1 receptor and, as such, mutations in this domain lead to a salient decrease in receptor−ligand affinity. In stark contrast, our data suggest that the SBDLII domain does not belong to the

Sigma1 receptor ligand binding site; accordingly, mutations in this protein domain exert only a marginal effect on ligand binding.

Sigma1 Receptor Residues Belonging to the C-Terminal End As mentioned in Section 1, Sigma1 receptor ligand binding is abrogated when more than 15 amino acids are removed from the C-terminal end of the protein (Kahoun and Ruoho 1992). Our group further investigated this aspect by deleting 7, 15, and 23 aa residues from the C-terminal end of the Sigma1 receptor and determined the affinity of the truncated receptors for (+)-pentazocine (Brune et al. 2014). As expected, elimination of 15 and 23 C-terminal amino acids resulted in the loss of (+)-pentazocine binding ability (Fig. 12). Also, removal of only seven residues from the receptor C-terminal end led to a considerable decrease in the level of (+)-pentazocine binding (specific binding 37%, Fig. 12). Again, these experimental data were consistent with in silico assays based on the 3D homology model of the Sigma1 receptor. While the major deletions resulted in a partially unfolded structure of the receptor missing a large portion of the ligand binding site, the affinity of the seven residue-truncated protein for (+)-pentazocine remained low, with a ΔG_{bind} value of −8.80 kcal/mol. The corresponding loss of 1.22 kcal/mol with respect to the WT receptor correlates with the 63% decrease in affinity reported by in vitro mutagenesis (Fig. 12). The seven deleted residues are not directly involved in ligand binding; however, the missing Tyr-Leu-Phe-Gly-Asn-Asp-Pro sequence results in a structural modification of the receptor that, like a domino effect, propagates along the protein backbone to the binding site. This overall configuration rearrangement directly affects three Sigma1 receptor residues most important for ligand binding, namely Arg119, Asp126, and Glu172. In particular, the interaction of Asp126 with (+)-pentazocine becomes less favorable by 1.54 kcal/mol with respect to the WT receptor, while Glu172 decreases its contribution by 1.68 kcal/mol.

Sigma1 Receptor Binding Site Distal Residues The last part of our Sigma1 receptor combined in vitro/in silico mutagenesis study was devoted to ascertain whether other residues could play a critical role in binding (+)-pentazocine. Thus, several alternative positions between the SBDLI and SBDLII receptor domains were mutated into alanine. Computer-based alanine mutagenesis results suggested that the Val145Ala and Phe146Ala mutants should result in minor or minimal changes in the protein binding site conformation, the estimated corresponding decrease in affinity being 0.13 kcal/mol, 0.60 kcal/mol, respectively. These predictions were confirmed by the corresponding specific binding values, as shown in Fig. 12. In contrast, changing the basic amino acid Arg175 and the two polar residues Ser125 and Thr127 into alanine was predicted to exert a moderate (Ser125Ala and Thr127Ala) to strong (Arg175Ala) influence on (+)-pentazocine binding, resulting in a decrease in receptor affinity of 1.45 kcal/mol, 1.24 kcal/mol, and 2.02 kcal/mol for the three residues, respectively. These in silico mutagenesis data were confirmed by experimental specific binding values of 49, 45, and 10%, as shown in Fig. 12. The Ser125Ala and Thr127Ala mutations transform the environment in the

proximity of the negatively charged Asp126 from polar to hydrophobic; this, in turn, increases the ADL of the involved salt bridges with (+)-pentazocine (4.76 and 4.69 Å for Ser125Ala and Thr127Ala, respectively) and, hence, weakens their strength. The case of the Arg175Ala mutant is more complex, as experiments detect a significantly reduced affinity of this mutant Sigma1 isoform for (+)-pentazocine (specific binding 10%, Fig. 12). The calculated ΔG_{bind} is 2.02 kcal/mol lower than that of the WT Sigma1 complex, although this residue is not directly involved in (+)-pentazocine binding. Importantly, however, the corresponding molecular dynamics simulation trajectory reveals that Arg175 forms a stable, bifurcated hydrogen bond with the side chains of Tyr120 and Arg114. These residues, in turn, stabilize the conformation of Tyr173 for productive binding via another direct hydrogen. The vanishing of all these interactions upon mutating Arg175 into alanine then explains the salient reduction of Sigma1 receptor mutant affinity for its agonist ligand.

3.3 Validation by Solution CD-NMR

In 2015, Ortega-Roldan and coworkers published a combined solution circular dichroism (CD)-nuclear magnetic resonance (NMR) study of a Sigma1 receptor construct in which only the first transmembrane domain and the eight-residue N-terminus have been removed (Ortega-Roldan et al. 2015). According to these authors, and independently of our published 3D homology Sigma1 receptor model, the secondary structure schematic of essentially the entire receptor was derived. In essence, the second transmembrane helix, TM2, was found to be composed of residues 91–107, which corresponds closely to the SBDLI. The cytosolic domain contains three α-helices, the third cytosolic helix cH3 (residues 81–85) being somewhat more mobile compared with the other two helices cH1 and cH2, and a modest increase in mobility of the GGW motif that joins this third cytosolic helical motif to the TM2 helix was observed.

Importantly, the experimentally determined secondary structure of the Sigma1 receptor strongly correlates with our 3D homology model, as shown in Fig. 14.

This work then constitutes the final and definitive experimental validation of the 3D homology model of the Sigma1 receptor in its monomeric form.

4 Does the Solution Structure of the Sigma1 Receptor Compare with Its Solid-State Conformation?

While writing this chapter, in early 2016 the first X-ray structure of the Sigma1 receptor was solved by Kruse and co-workers (Schmidt et al. 2016). Quite surprisingly, the solid-state structure of the receptor looks completely different from the one independently proposed by our group (Laurini et al. 2011) and solved by NMR by Schnell and colleagues (Ortega-Roldan et al. 2015), as discussed above. Indeed,

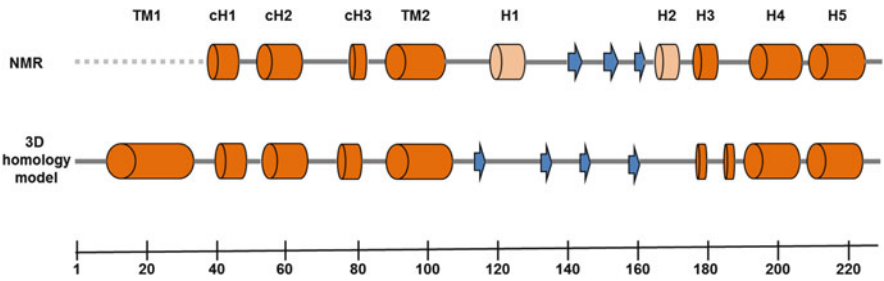

Fig. 14 (*Top*) Secondary structural motifs of the Sigma1 receptor as determined by solution NMR (Ortega-Roldan et al. 2015) and (*bottom*) of the validated 3D homology model. α-helical domains are portrayed as orange cylinders, β-sheet motifs are shown as *blue arrows*, while *gray lines* represent coils and unstructured regions. In the *top panel* sequence, the TM1 region is not reported as it was missing in the Sigma1 construct used in the experiment. Ortega-Roldan et al. (2015) indicated the presence of two small helices (H1 and H2, *light orange cylinders*) involving residues 121–130 and 168–174, respectively. However, these authors declare that these residues exhibited a mixed propensity for helical and extended conformation. Therefore, given the approximation of the experimental technique involved, the structure of the Sigma1 receptor region including residues 120–180 agrees in both models

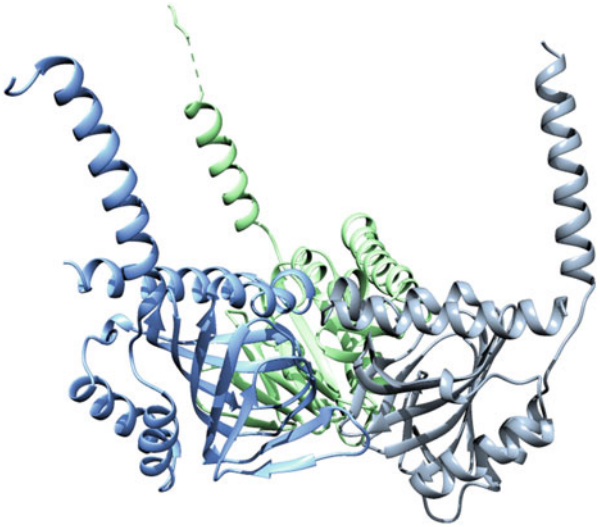

Fig. 15 Rendering of the solved X-ray structure of the Sigma1 receptor (PDB file: 5HK1.pdb). The three receptor chains are colored in *blue*, *green,* and *light gray*, respectively

according to Kruse himself in different public interviews (Kruse 2016), the receptor "looks a lot different from what it was supposed to look like" (see Fig. 15).

First, in contrast to the structure proposed on the basis of biochemical, molecular, in silico, and NMR data, the X-ray structure of the protein features only one instead of two transmembrane domains. Next, according to Kruse, the solid-state

structure of the receptor has a "strange triangular fold," and is composed by a trimeric structure consisting of three identical copies of itself. Third, it docks unusually "half in and half out" of the membrane, rather than being completely embedded within it or floating in the cytoplasm. To support the peculiarity of the Sigma1 receptor solid-state structure, Kruse concluded his interview (Kruse 2016) by stating, "It's unlike any other protein structure we've ever seen."

So, some questions naturally arise: Which is the true structure of the Sigma1 receptor? Should we rely on the solution-solved or the X-ray-solved structure? How can such a significant difference between the two structures be explained?

In our opinion, the first question still has a long way to go before the field will be able to provide an unequivocal answer. Yet, some arguments can be proposed in response to the latter two questions. As discussed in the preceding sections of this chapter, our 3D homology model is supported by experimental ligand binding studies, mutagenesis studies, and most importantly, by the NMR studies published by Schnell and colleagues. Given the perfect match between the in silico derived model and the NMR solved structure (see Fig. 14), it is difficult to believe that the identity of the two (homology and NMR) receptor structures is a matter of pure serendipity.

The significant differences between NMR and crystal structures of membrane proteins may arise from a combination of differences in several factors, such as (1) structure determination methods, (2) environment of a packed crystal versus solution, (3) the hydrophobic environment of the membrane bilayer, (4) the membrane mimetic, and (5) specific lipid/detergent types used. The conditions adopted in the two procedures (in silico/NMR and solid state) were indeed different (micelles vs. nanodiscs and different membrane-mimetic compositions), coupled to the different solvent, temperature and protein expression/purification conditions, might result in different protein structures. In our opinion, to support the validity of the X-ray structure compared to the one derived from solution studies, it would have been informative to perform X-ray experiments under the conditions adopted for NMR/in silico experiments and vice versa (i.e., same membrane shape – micelles – and same membrane composition).

It is noteworthy that Kruse himself highlights the difficulty in explaining how a ligand can access the binding site in his model. Quoting the author: "Given the highly occluded structure of the binding pocket, it remains unclear how ligands enter and exit this site" (Schmidt et al. 2016).

There are many publications that support the two-transmembrane domain structure of the Sigma1 receptor which, of note, were not discussed by Kruse in the light of his proposed protein model.

Indeed, the two-transmembrane domain model of the Sigma1 receptor was originally proposed based on biochemical and molecular biology studies. Here are some examples. The first study to support this view was by Aydar et al. (2002). According to this study, data derived from N- and C-terminal GFP-Sigma1 receptor expression in *Xenopus* oocytes support the conclusion that the Sigma1 receptor contains two transmembrane domains, resulting in an extracellular loop of approximately 50 amino acids and an intracellular C-terminal domain of approximately 120 amino acids.

Next, according to the work of Hayashi and Su (2007) in Chinese hamster ovary cells, the topological model of the Sigma1 receptor supports the two-transmembrane model initially proposed by Aydar et al. (2002). Hayashi and colleagues combined immunocytochemistry and protease protection assays to conclude that the Sigma1 receptor possesses two transmembrane domains with amino acid residues 29–92 projecting into the cytosol and the segment starting from residue 113 to the end of the C-terminus comprising a long ER lumenal domain (Hayashi and Su 2007).

In vitro oligomeric forms of the maltose-binding protein (MBP)–Sigma1 fusion protein (MBP–S1R) (tetramer/hexamer/octamer) have been reported which depend on a helix–helix dimerization GXXXG sequence in the putative second transmembrane domain (Gromek et al. 2014).

Ruoho and colleagues have very recently devised a further experiment according to which the N- and C-termini of the Sigma1 receptor are on the same side of the membrane, again supporting the two-transmembrane structure of the receptor (Ruoho, personal communication).

In conclusion, in the light of the above arguments and pending more experimental data on the structure of the Sigma1 receptor, we propose that the protein adopts different structures under solid (revealed by the crystal structure) and solution states (revealed by the in silico and NMR models).

5 Conclusions and Future Work

This chapter reviews our current knowledge of the secondary and 3D structure of the Sigma1 receptor. There is still much work to be done in the field of Sigma1 receptor structural biology. For example, our laboratory is currently working to model the structure of the putative dimeric/oligomeric forms of the Sigma1 receptor, as shown in Fig. 16.

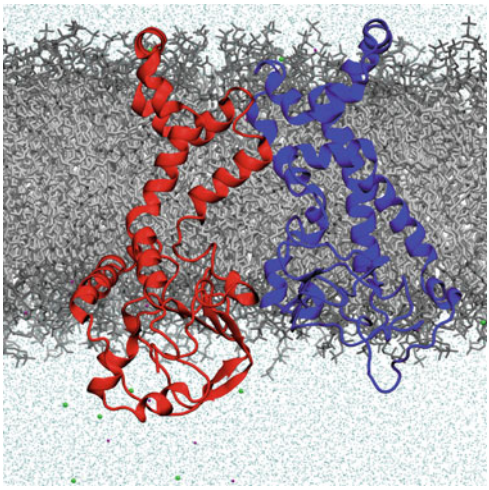

Fig. 16 Equilibrated molecular dynamics simulation of the dimeric model of the Sigma1 receptor. One receptor chain is portrayed as a *red ribbon*, the other as a *blue* one. Membrane molecules are evidenced as *gray sticks*. Water molecules, ions and counterions are shown as *light blue* transparent *small spheres*

The road to understanding the structural biology of the Sigma1 receptor is defined but insufficiently cobbled. The challenge of resolving the discrepancies in the field are undeniably worth all possible efforts.

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Medicinal Chemistry of σ_1 Receptor Ligands: Pharmacophore Models, Synthesis, Structure Affinity Relationships, and Pharmacological Applications

Frauke Weber and Bernhard Wünsch

Contents

Abstract

In the first part of this chapter, we summarize the various pharmacophore models for σ_1 receptor ligands. Common to all of them is a basic amine flanked by two hydrophobic regions, representing the pharmacophoric elements. The development of computer-based models like the 3D homology model is described as

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well as the first crystal structure of the σ_1 receptor. The second part focuses on the synthesis and biological properties of different σ_1 receptor ligands, identified as **1**-**9**. Monocyclic piperazines **1** and bicyclic piperazines **2** and **3** were developed as cytotoxic compounds, thus the IC_{50} values of cell growth and survival inhibition studies are given for all derivatives. The mechanism of cell survival inhibition, induction of time-dependent apoptosis, of compound **ent-2a** is discussed. Experimentally determined σ_1 affinity shows good correlation with the results from molecular dynamics simulations based on a 3D homology model. Spirocyclic compounds **4** and **5** represent well-established σ_1 receptor ligands. The homologous fluoroalkyl derivatives **4** have favorable pharmacological properties for use as fluorinated PET tracers. The (S)-configured fluoroethyl substituted compound **(S)-4b** is under investigation as PET tracer for imaging of σ_1 receptors in the brain of patients affected by major depression. 1,3-Dioxanes **6c** and **6d** display a very potent σ_1 antagonist profile and the racemic 1,3-dioxane **6c** has high anti-allodynic activity at low doses. The arylpropenylamines **7** are very potent σ_1 receptor ligands with high σ_1/σ_2 selectivity. The top compound **7g** acts as an agonist as defined by its ability to potentiate neurite outgrowth at low concentrations. Among the morpholinoethoxypyrazoles **8**, **8c** (known as S1RA) reveals the most promising pharmacokinetic and physicochemical properties. Due to its good safety profile, **8c** is currently being investigated in a phase II clinical trial for the treatment of neuropathic pain. The most potent ligand **9e** of 3,4-dihydro-2(1H)-quinolones **9** shows promising anti-nociceptive activity in the formalin test.

Keywords
Pharmacological data • Structure (σ_1) affinity relationships • Synthesis • σ_1 Receptor ligands • σ_1 Receptor pharmacophore models

1 Introduction

The sigma-1 (σ_1) receptor is a membrane-bound protein distributed in the central nervous system and in peripheral organs like heart, kidney, and liver (Weissman et al. 1988; Samovilova et al. 1988; Ela et al. 1994). The σ_1 receptor is mainly localized at the endoplasmic reticulum and the mitochondria-associated membranes. Ruoho et al. have shown that the σ_1 receptor consists of two transmembrane regions connected by a loop. Both *C*- and *N*-terminus are located extracellularly or in the ER lumen (Chu and Ruoho 2016). Two additional hydrophobic regions of the σ_1 receptor were identified by Fontanilla et al. named steroid binding domain-like regions (SBDL I and II). With the help of *N*-substituted photoaffinity labels it was shown that the SBDL I overlaps with one of the two transmembrane regions of the σ_1 receptor forming the ligand-binding domain together with the SBDL II (Ruoho et al. 2012). σ_1 Receptors were shown to take part in the regulation of ion channels (e.g., K^+ and Ca^{2+}) and in the modulation of neurotransmitter systems (Lupardus et al. 2000; Hong and Werling 2000; Hayashi

and Su 2007). In the brain, the σ_1 receptor is particularly well expressed in areas associated with memory and emotion (Mash and Zabetian 1992). Steroids like progesterone (Su et al. 1988; Schwarz et al. 1989) and *N*,*N*-dimethyltryptamine (Fontanilla et al. 2009) were previously discussed to be endogenous ligands but their σ_1 receptor binding affinities are low compared with those of sphingosines showing high affinity in the low-nanomolar range (Ruoho et al. 2012). Since many centrally active drugs show high σ_1 affinity, σ_1 receptors represent promising targets for the research and development of drugs to treat several neurological or neuropsychiatric disorders like depression, psychosis, and cocaine abuse (Hascoet et al. 1995; Matsumoto et al. 2001; Sharkey et al. 1988; Bermack and Debonnel 2001; Ishikawa et al. 2007; Skuza and Rogoz 2006). The fact that many human cancer cell lines show up-regulated levels of σ_1 receptors brought them into focus for the development of new antitumor drugs and cancer diagnostics (Hashimoto and Ishiwata 2006). Based on various studies, Chen and Pasternak postulated that the σ_1 receptor functions as an endogenous anti-opioid receptor system (Chien and Pasternak 1993). By investigation of different σ_1 receptor ligands in animal models it was shown that σ_1 agonists inhibit morphine-induced analgesia whereas σ_1 antagonists potentiate opioid induced analgesia (Chien and Pasternak 1994, 1995). The fact that σ_1 knockout mice show reduced pain response in the formalin test but not hypersensitivity after treatment with capsaicin lead to interest in σ_1 receptors as a target in the treatment of neuropathic pain (Entrena et al. 2009).

For the development of new potent σ_1 receptor ligands with high affinity several pharmacophore models have been developed and optimized. Herein the pharmacophore models reported so far are summarized and compared with respect to existing ligands.

2 Pharmacophore Models

In 1994, Glennon et al. reported a two-dimensional pharmacophore model based on deconstruction–reconstruction analysis of different flexible σ_1 receptor ligands. In this model, two hydrophobic regions flanking a basic amine represent the pharmacophoric elements required for high σ_1 affinity. A distance of 6–10 Å between the amine moiety and the primary hydrophobic region and of 2.5–3.9 Å between the amino group and the secondary hydrophobic region provides optimal binding conditions. The amine could be of primary, secondary, or tertiary nature. In case of a tertiary amine, only small substituents are allowed, whereas the amine could also be part of a ring system (e.g., piperazine ring). The primary hydrophobic region tolerates sterically demanding residues whereas the secondary region favors smaller substituents like a three-carbon chain. As the two hydrophobic binding pockets of the σ_1 receptor tolerate bulky groups, the size of substituents can vary slightly without decreasing binding affinity (Fig. 1) (Glennon et al. 1994; Glennon 2005).

Laggner et al. presented in 2005 the first computer-aided three-dimensional pharmacophore model (Fig. 2) based on 23 structurally very different σ_1 ligands.

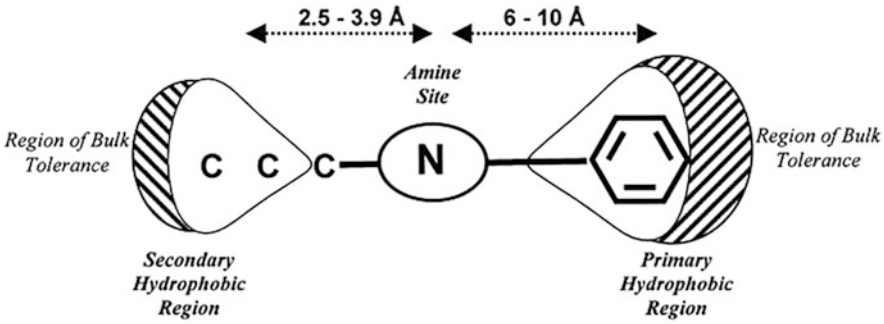


Fig. 1 Pharmacophore model of Glennon (2005)

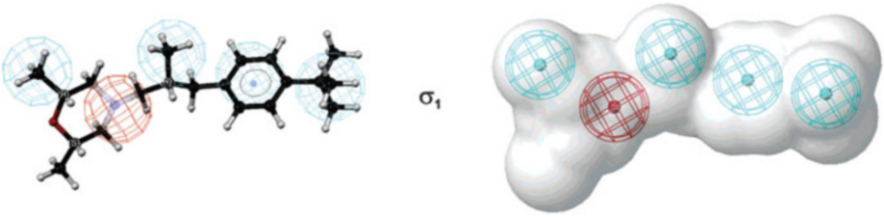


Fig. 2 3D-σ_1-Pharmacophore model of Laggner et al. (2005) *red*: positive ionizable group; *blue*: hydrophobic regions

The pharmacophoric elements consist of a positive ionizable group like an amine and four hydrophobic features. The calculated distances between the pharmacophoric elements are in good agreement with the results obtained by Glennon et al. (1994).

In 2009, Zampieri et al. designed another computer-based model containing five pharmacophoric elements (Fig. 3) (Zampieri et al. 2009). The model included three hydrophobic areas in total (depicted in blue and pink), whereby two of them should have aromatic character (blue). A basic center (red) is located at a distance of 7.01 and 8.50 Å from the aromatic moieties and at a distance of 3.58 Å from the further hydrophobic elements. These distances are comparable to those postulated in the models of Glennon and Laggner. Additionally, the Zampieri model established an H-bond acceptor function, which was already defined in a pharmacophore model by Gilligan et al. This model was published in the early 1990s and did not differentiate between σ_1 and σ_2 ligands (Gilligan et al. 1992).

In 2011, Laurini et al. published the first computer-based 3-dimensional (3D) homology model of the σ_1 receptor. For the identification of a reliable ligand-binding domain, results of docking studies, mutagenesis studies, structure–affinity-relationship studies, and pharmacophore models were combined. The validation of the homology model was implemented by docking studies of well-known σ_1 ligands at the postulated binding site of the receptor, calculation of free binding

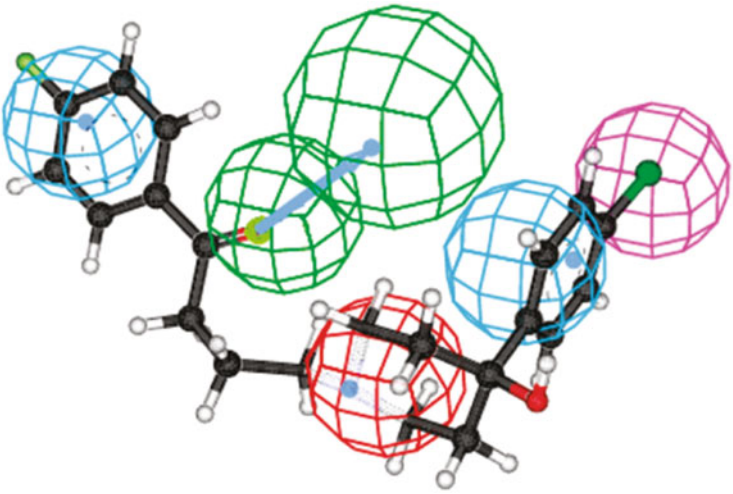


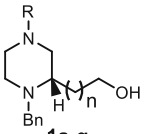
Fig. 3 Pharmacophore model of Zampieri et al. (2009) *red*: basic center; *green*: H-bond acceptor; *blue*: aromatic hydrophobic area; *pink*: hydrophobic area

energy, and comparison with the experimentally determined σ_1 affinities of these ligands (Laurini et al. 2011, 2012).

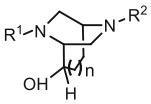
Schmidt et al. have just published a crystal structure of the σ_1 receptor for the first time (Schmidt et al. 2016). This structure was determined in complex with two different σ_1 receptor ligands, PD144418 and 4-IBP. Contrary to the early findings of Fontanilla and Ruoho (Ruoho et al. 2012) as well as Aydar et al. only one transmembrane domain of the σ_1 receptor was found in the crystal structure (Schmidt et al. 2016). This contradicts also the solution nuclear magnetic resonance (NMR) results of Ortega-Roldan et al. (2015) which closely match the findings of the 3D homology model of Laurini et al. (2011).

3 Ligands Introduction

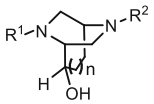
In the literature, a great variety of σ_1 receptor ligand classes are reported. These classes include piperazines **1**, bicyclic compounds of type **2** and **3**, spirocyclic compounds **4** and **5**, 1,3-dioxanes **6**, arylalkenylamines **7**, morpholinoethoxypyrazoles **8**, and 3,4-dihydro-2(1*H*)-quinolones **9** (Fig. 4). The synthesis and the pharmacological properties of these ligands are presented herein.



1a-g



2a-f

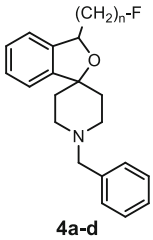


3a-f

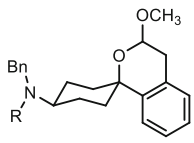
	n	R	$K_i(\sigma_1)$
1a	0	PMB	12 nM
1b	1	PMB	20 nM
1c	1	Me	> 1000 nM
1d	1	$C_6H_{11}CH_2$	4.2 nM
1e	1	$C_6H_{11}CH_2$	4.7 nM
		Bn = 2-NaphCH_2	
1f	2	$C_6H_{11}CH_2$	275 nM
1g	3	$C_6H_{11}CH_2$	52 nM

	n	R^1	R^2	$K_i(\sigma_1)$
2a	0	$C_6H_{11}CH_2$	$PhCH_2$	4.8 nM
2b	0	$C_6H_{11}CH_2$	1-NaphCH_2	8.0 nM
2c	0	$C_6H_{11}CH_2$	4-Ph-$PhCH_2$	9.8 nM
2d	1	$C_6H_{11}CH_2$	$PhCH_2$	6.0 nM
2e	1	PMB	$PhCH_2$	125 nM
2f	2	PMB	$PhCH_2$	298 nM

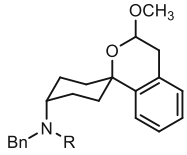
	n	R^1	R^2	$K_i(\sigma_1)$
3a	0	$C_6H_{11}CH_2$	$PhCH_2$	6.9 nM
3b	0	$C_6H_{11}CH_2$	1-NaphCH_2	7.1 nM
3c	0	$C_6H_{11}CH_2$	4-Ph-$PhCH_2$	9.8 nM
3d	1	$C_6H_{11}CH_2$	$PhCH_2$	6.0 nM
3e	1	PMB	$PhCH_2$	6.5 nM
3f	2	PMB	$PhCH_2$	25 %[a]



4a-d



cis-**5a-d**



trans-**5a**

	n	$K_i(\sigma_1)$
4a	1	0.74 nM
4b	2	0.59 nM
4c	3	1.4 nM
4d	4	1.2 nM

	R	$K_i(\sigma_1)$
cis-**5a**	CH_3	24 nM
cis-**5b**	C_2H_5	107 nM
cis-**5c**	C_5H_{11}	>1000 nM
cis-**5d**	$PhCH_2$	>1000 nM

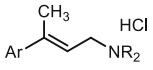
	R	$K_i(\sigma_1)$
trans-**5a**	CH_3	43 nM

6a-d

	R^1	R^2	$K_i(\sigma_1)$
(*S*,*R*)-**6a**	H	H	>10000
(*R*,*R*)-**6b**	CH_3	H	>10000
(*S*,*R*)-**6c**	H	$PhCH_2$	6.0 nM
(*R*,*R*)-**6d**	CH_3	$PhCH_2$	17 nM

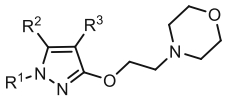
	R^1	R^2	$K_i(\sigma_1)$
(*R*,*S*)-**6a**	H	H	>10000
(*S*,*S*)-**6b**	CH_3	H	>10000
(*R*,*S*)-**6c**	H	$PhCH_2$	50 nM
(*S*,*S*)-**6d**	CH_3	$PhCH_2$	11 nM

Fig. 4 Representative σ_1 receptor ligands of different compound classes. Inhibition of σ_1 receptor radioligand binding at 1 μM concentration of test compound. *PMB* *p*-methoxybenzyl; *Naph* naphthyl



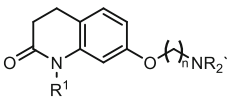
7a-f·HCl

	Ar	NR_2	$K_i(\sigma_1)$
7a	4-Ph-$PhCH_2$	piperidine	0.86 nM
7b	2-Naph	piperidine	0.97 nM
7c	4-Ph-$PhCH_2$	4-benzylpiperidine	7.0 nM
7d	2-Naph	4-benzylpiperidine	23 nM
7e	4-Ph-$PhCH_2$	morpholine	12 nM
7f	Ph	morpholine	>1000 nM



8a-f

	R^1	R^2	R^3	$K_i(\sigma_1)$
8a	*tert*-butyl	H	H	>1000 nM
8b	4-chlorophenyl	H	H	18 nM
8c	2-Naph	CH_3	H	17 nM
8d	2-Naph	CH_3	CH_3	139 nM
8e	3,4-dichlorophenyl	CH_3	CH_3	9.4 nM
8f	3,4-dichlorophenyl	CH_3	$C(C=O)CH_3$	741 nM



9a-i

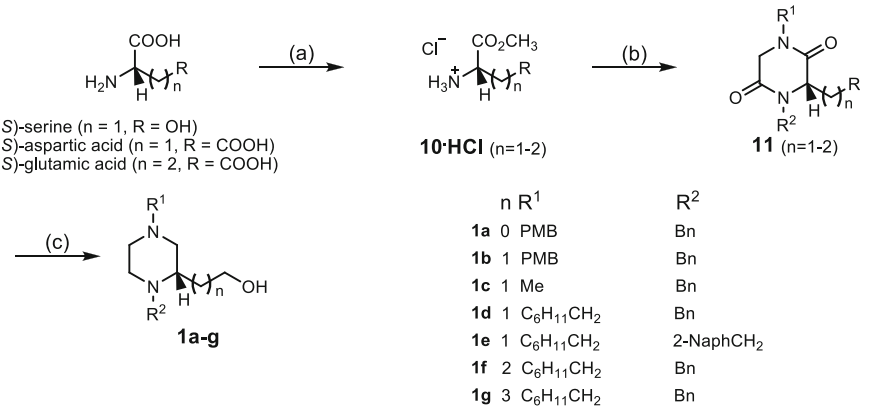
	R^1	n	NR_2`	$K_i(\sigma_1)$
9a	$PhCH_2$	2	morpholine	> 2000 nM
9b	$PhCH_2$	3	morpholine	15 nM
9c	H	3	piperidine	89 nM
9d	CH_3	3	piperidine	34 nM
9e	$PhCH_2$	3	piperidine	1.84 nM
9f	4-F-Ph-CH_2	3	piperidine	1.22 nM
9g	$PhCH_2$	4	morpholine	60 nM
9h	$PhCH_2$	5	morpholine	88 nM
9i	$PhCH_2$	6	morpholine	682 nM

Fig. 4 (continued)

4 Homologous 2-Piperazinealkanols

Piperazines **1** with different hydroxyalkyl side chains represent well-established σ_1 receptor ligands. A broad structure affinity relationship study was performed based on 2-hydroxyethyl substituted piperazines with high σ_1 affinity and their larger and smaller homologs bearing hydroxypropyl or hydroxymethyl side chains. The cytotoxic activity against human cancer cell lines was tested by in vitro cell survival assays (Weber et al. 2014; Holl et al. 2012; Bedurftig and Wunsch 2004).

(*S*)-Serine, (*S*)-aspartic acid, and (*S*)-glutamic acid as enantiomerically pure amino acids of nature's chiral pool were used for the synthesis of homologous piperazinealkanols **1a-g**. The first reaction step includes the esterification of the particular amino acid. The dioxopiperazines **11** were prepared from the aminoesters **10**·HCl in a three-step reaction sequence consisting of reductive alkylation, chloroacetylation, and ring closure with different primary amines. Reduction with



Scheme 1 Synthesis of homologous 2-piperazinealkanols **1**. Reagents and reaction conditions: (**a**) $(H_3C)_3SiCl$, CH_3OH, room temperature (rt), 16 h; (**b**) (1) Ph-CH=O, NEt_3, CH_2Cl_2, rt, 16 h; (2) $NaBH_4$, CH_3OH, 0 °C, 40 min; (3) $ClCH_2COCl$, NEt_3, CH_2Cl_2, rt, 2.5 h; (4) R^1-NH_2, NEt_3, CH_3CN, rt, 16 h–3 d; (**c**) $LiAlH_4$, THF, reflux, 16 h. *PMB* *p*-methoxybenzyl, *2-NaphCH*$_2$ 2-naphthyl (Weber et al. 2014; Holl et al. 2012; Bedurftig and Wunsch 2004)

$LiAlH_4$ led to the piperazinealkanols **1a-g** (Scheme 1). Reduction of the ester moiety of **11d** ($n = 1$, R = CO_2CH_3) followed by Wittig reaction of the resulting aldehyde with methyl(triphenylphosphoranylidene)acetate and subsequent reduction of the α,β-unsaturated ester led to the homologous hydroxybutyl piperazine **1g** (Weber 2012).

The σ_1 and σ_2 affinity of hydroxyalkyl piperazines **1a-g** was tested with tissue membrane preparations of animal origin (guinea pig brain, rat liver). Selected ligands (**1d-g**) were also assayed with membrane preparations bearing human σ_1 receptors to evaluate ligand-binding affinity towards σ_1 receptors from different species (Table 1).

For a high σ_1 affinity, the length of hydroxyalkyl side chain and the size of the residues at both *N*-atoms are of particular importance. Short side chains like hydroxymethyl and hydroxyethyl are well-tolerated by the σ_1 receptor leading to K_i values in the range of 4–20 nM. The σ_1 affinity of the hydroxypropyl piperazines is more than tenfold lower (e.g., **1f**, $K_i = 275$ nM). The extension of the side chain by another methylene moiety in case of hydroxybutyl piperazine **1f** leads to an increased σ_1 affinity, but the K_i value of 52 nM remained higher than the K_i value measured for hydroxymethyl and hydroxyethyl derivatives. In accordance with the pharmacophore model of Glennon postulating two hydrophobic regions, the *N*-methyl substituted piperazine **1c** does not show high σ_1 receptor affinity. The affinity increases by the introduction of a larger residue such as cyclohexylmethyl or *p*-methoxybenzyl group.

The hydroxyethyl derivatives show almost the same σ_1 affinity as the hydroxymethyl derivatives, but show reduced σ_2 affinity than hydroxyethyl piperazines. Regarding the σ_1/σ_2 selectivity, it becomes clear that the hydroxyethyl

Table 1 Inhibition of cancer cell growth by piperazinealkanols **1a-g** compared with σ_1 and σ_2 binding affinity

	σ_1 (gp)[a] K_i [nM] ± SEM	σ_2 (rat)[b] K_i [nM] ± SEM	σ_1 (hu)[c] K_i [nM] ± SEM	σ_1/ σ_2 selectivity (ratio)	$\sigma_1 K_i$ (calc) [nM]	IC_{50} [μM] RT4[d]	IC_{50} [μM] 5637[e]	IC_{50} [μM] A-427[f]	IC_{50} [μM] LCLC-103H[g]	IC_{50} [μM] DAN-G[h]	IC_{50} [μM] MCF-7[i]	IC_{50} [μM] RPMI 8226[j]
1a	12 ± 1.4	70 ± 10	–	6	–	–	–	–	–	–	–	–
1b	20 ± 6.0	>1,000	–	50	–	–	–	–	–	–	–	–
1c	28%[k]	27%[k]	–	–	–	>20	>20	>20	>20	>20	>20	>20
1d	4.2 ± 1.1	116[l]	21 ± 4	28	35	>20	>20	14 ± 5	>20	>20	6.8 ± 8.4	–
(*ent*)-1d	1.9 ± 0.4	60 ± 11	23 ± 8	32	–	–	>20	>20	>20	>20	–	>20
1e	4.7 ± 1.8	69 ± 24	6.8 ± 2.3	15	11	>20	2.1 ± 1.9	2.5 ± 2.6	>20	>20	15 ± 2	7.3 ± 3.9
1f	275[l]	690[l]	71[l]	3	–	>20	8.0 ± 5.6	>20	>20	>20	>20	>20
1 g	52[l]	348[l]	54 ± 26	7	–	>20	6.0 ± 4.0	9.6 ± 5.1	>20	>20	>20	>20

[a]Guinea pig brain (gp)
[b]Rat liver (rat)
[c]RPMI 8226 human cancer cell line (hu)
[d]RT4 bladder cancer cell line
[e]Bladder cancer
[f]Small cell lung cancer
[g]Large cell lung cancer
[h]Pancreas cancer
[i]Breast cancer
[j]Multiple myeloma
[k]Inhibition of radioligand binding at 1 μM concentration of test compound (% inhibition)
[l]Result from one measurement. All other results are from three independent experiments

derivatives are more than tenfold selective for the σ_1 over the σ_2 receptor. The PMB-substituted compound **1b** provides the best selectivity in this set of compounds with a σ_2 affinity of >1,000 nM. As a result of further chain extension, the σ_2 affinity increases leading to decreased selectivity (**1f**, K_i (σ_2) = 690 nM, **1g**, K_i (σ_2) = 348 nM).

The σ_1 affinity of **1d-g** measured with membrane preparations from a human cancer cell line (RPMI 8226) is slightly reduced compared to the affinity measured with the guinea pig brain membrane preparations. Because the same trend was found for the reference compounds haloperidol and (+)-pentazocine it can be assumed that the results of both assays are well comparable.

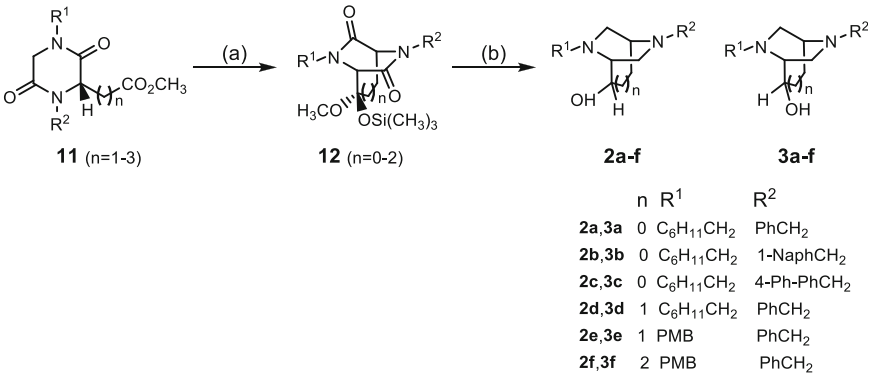
As the enantiomer of **1d** prepared in the same manner from (*R*)-aspartic acid shows the same σ_1 affinity, it could be assumed that the stereochemistry has only negligible influence on σ_1 receptor affinity and selectivity over the σ_2 subtype ($K_i(\sigma_1)$ = 1.9 nM, σ_1/σ_2 = 32).

The σ_1 affinity obtained with human receptor preparations was supported by docking of the ligands in the putative binding site of the 3D σ_1 receptor homology model. The calculated free binding energies are in good accordance with their recorded affinities towards the σ_1 receptor. For the most potent human σ_1 receptor ligand **1g** ($K_{i,exp.}$ = 6.8 nM) a ΔG_{bind} of -10.85 ± 0.36 kcal/mol was calculated which corresponds to an estimated $K_i(\sigma_1)_{calcd}$ value of 11.2 nM, consistent with the experimentally determined K_i values (Weber et al. 2014).

The cell growth inhibition potential of piperazinealkanols **1c-g** was tested in seven human tumor cell lines. The potent σ_1 receptor ligand **1e** inhibited the growth and survival of the bladder cancer cell line 5637, the small cell lung cancer cell line A427, and the multiple myeloma cell line RPMI 8226 in the low micromolar range. Even at high concentrations (20 μM) of **1e**, a growth inhibition activity could not be found for the bladder cancer cell line RT4, the large cell lung cancer LCLC-103H, and the pancreas cancer cell line DAN-G. Additionally only low activity was found for the breast cancer cell line MCF-7, indicating a selective mechanism of growth and survival inhibition. Further investigation of the mechanism associated with the inhibitory activity of **1e** was performed with RPMI 8226 cells and revealed an increase in the amount of early apoptotic cells after 48 h compared to the untreated control.

5 Bicyclic Piperazines

In order to investigate the influence of conformational restriction on σ_1 receptor affinity bicyclic compounds of type **2** and **3** with diazabicycloalkan scaffold were designed by intramolecular connection of the 2-hydroxyalkyl side chain of piperazines **1** with *C*-5 of the piperazine ring. Propano- and butano-bridged homologs of **2** and **3** with diazabicyclo[3.2.2]nonane and diazabicyclo[4.2.2]decane scaffold were synthesized by an expansion of the ethano-bridge by one or two



Scheme 2 Synthesis of bicyclic diazabicycloalkanols **2** and **3**. Reagents and reaction conditions: (**a**) $n = 1$: NaHMDS, THF, −78 °C, 40 min, then $(H_3C)_3SiCl$, −78 °C, 1 h, then rt, 2 h (Weber et al. 2016); $n = 2$: LiHMDS, THF, −78 °C, 30 min, then $(H_3C)_3SiCl$, −78 °C, 0.5 h, then rt, 3 h (Geiger et al. 2007); $n = 3$: LiHMDS, THF, −78 °C, 30 min, then $(H_3C)_3SiCl$, −78 °C, 2 h, then rt, 0.5 h (Sunnam 2010); (**b**) $n = 0$: 1. 0.5 M HCl, THF, rt, 16 h; 2. $LiAlH_4$, THF, reflux, 16 h (Weber et al. 2016); $n = 1$: 1. *p*-TosOH, THF, H_2O, rt, 16 h; 2. $LiBH_4$, THF, −90 °C, 3.5 h; 3. $LiAlH_4$, THF, reflux, **16 h** (Geiger et al. 2007) $n = 2$: 1. *p*-TosOH, THF, H_2O, rt, 16 h; 2. $LiBH_4$, THF, −90 °C, 2.5 h; 3. $LiAlH_4$, THF, reflux, 15 h (Sunnam 2010)

methylene moieties to elucidate the effect of bridge size on σ_1 receptor affinity (Geiger et al. 2007; Weber et al. 2016; Sunnam 2010).

Homologous dioxopiperazines **11** ($n = 1$–3) with different substitution pattern of the *N*-atoms were used as starting material for the synthesis of bicyclic compounds **2** and **3** (Scheme 2). The dioxopiperazine **11** ($n = 3$) was synthesized from racemic-2-aminoadipic acid in the same manner as explained for dioxopiperazines **11** ($n = 1,2$) in Scheme 1. The mixed methyl silyl ketals **12** were obtained by Dieckmann analogous cyclization of **11**. The Dieckmann analogous cyclization gave only low yields for the dioxopiperazines **11** ($n = 1$) with acetate side chain due to the rigidity of the resulting products **12** ($n = 0$). The (*R*)-configuration of the ketalic center of **12** was shown by X-ray crystal structure analysis (Holl et al. 2008). Hydrolysis and reduction of **12** led to the bicyclic alcohols **2** and **3**. The enantiomers *ent*-**2** and *ent*-**3** were obtained starting with (*R*)-configured amino acids.

In piperazinealkanols **1** the hydroxyalkyl side chain can adopt several conformations and, moreover, the piperazine ring can adopt two conformations, leading to an axial or equatorial orientation of the side chain resulting in different distances between the pharmacophoric elements. In the bicyclic alcohols **2** and **3** the additional bridge over the piperazine ring reduces the flexibility of the ring system and its hydroxyalkyl side chain. As a result of conformational restriction, the pharmacophoric elements are fixed in a defined arrangement minimizing the loss of entropy during binding and thus increasing the overall free binding energy.

The σ_1 and σ_2 receptor affinity of the bicyclic alcohols **2** and **3** was determined with tissue membrane preparations from guinea pig brain (for σ_1) and rat liver (for

σ_2). Compounds **2a-d** and **3a-d** were also tested against human σ_1 receptors from multiple myeloma RPMI 8226 cell line membrane preparations (Geiger et al. 2007; Weber et al. 2016).

Almost all cyclohexylmethyl substituted compounds **2a-d** and **3a-d** show high σ_1 receptor affinity with K_i values in the low-nanomolar range. The only exceptions are *ent*-**2a** and **3c**, both with a K_i value of 23 nM. The extension of the ethano-bridge of **2a-c** and **3a-c** by a methylene moiety does not influence σ_1 receptor affinity since the propane-bridged homologs **2d**,**e** and **3d**,**e** show approximately the same affinity. Only **2e** and *ent*-**2e** show K_i values in the three-digit nanomolar range ($K_i(\sigma_1) = 125$ and 118 nM). However, the introduction of a second methylene moiety leads to a salient decrease in σ_1 receptor affinity, which implies that butano-bridged diazabicycloalkanols **2f** and **3f** are not tolerated by the σ_1 receptor.

The stereochemistry has only low impact on σ_1 receptor affinity since all four stereoisomers **2a,** *ent*-**2a**, **3a,** and *ent*-**3a** show the same σ_1 receptor affinity. However, in case of PMB-substituted derivatives, **2e** and *ent*-**2e** show lower σ_1 receptor affinity than **3e** and *ent*-**3e**.

The σ_1 affinity determined with human σ_1 receptor material is in good accordance with the σ_1 affinity obtained with σ_1 receptors from guinea pig brain.

Compared with the flexible hydroxyethyl piperazines **1b**, **1d,** and **1g**, the corresponding ethano-bridged piperazines **2a-c** and **3a-c** reveal the same σ_1 receptor affinity. However, the conformational restriction of the hydroxypropyl piperazines **1d** led to increased σ_1 receptor affinity of **2e** and **3e**. That is due to the higher flexibility of the hydroxypropyl piperazines **1d,e** compared to their shorter hydroxyethyl homologs **1a-d**. This is not valid for the butano-bridged piperazines **2f** and **3f** which display very low σ_1 affinity, indicating that the bridge size is too bulky for the binding pocket. Obviously the size of the butano-bridge outweighed its positive effect of conformational restriction.

The σ_1/σ_2 selectivity varies from low preference for the σ_1 receptor (**2c**: $\sigma_1/\sigma_2 = 2$) up to high selectivity for the σ_1 receptor (**3d**: $\sigma_1/\sigma_2 = 178$). The PMB-substituted derivative *ent*-**3e** ($\sigma_1/\sigma_2 = 227$) showed the highest σ_1/σ_2 selectivity.

The bicyclic compounds **2a-d** and **3a-d** were docked into the binding site of the 3D homology model to determine the free binding energies. Figure 5 illustrates the identified interactions between the high affinity compound *ent*-**3a** ($K_i(\sigma_{1human}) = 1.6$ nM) and the human σ_1 receptor (Weber et al. 2016).

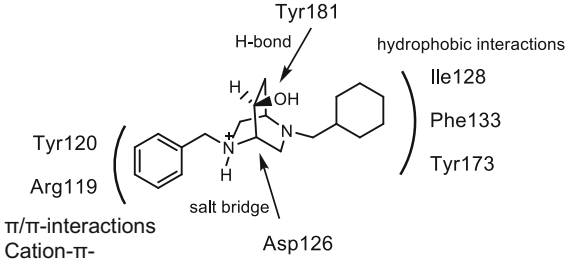


Fig. 5 Interactions between *ent*-3a and amino acids of the binding site in the 3D homology model of the human σ_1 receptor

Table 2 Pharmacological data for diazabicycloalkanols **2** and **3**

	σ_1 (gp)[a] K_i [nM] ± SEM	σ_2 (rat)[b] K_i [nM] ± SEM	σ_1 (hu)[c] K_i [nM] ± SEM	σ_1/σ_2 selectivity	$\sigma_1 K_i$ (calc) [nM]	IC_{50} [μM] RT4[d]	IC_{50} [μM] 5637[e]	IC_{50} [μM] A-427[f]	IC_{50} [μM] LCLC-103H[g]	IC_{50} [μM] MCF-7[h]
2a	4.8 ± 0.7	36 ± 9.0	3.2 ± 0.4	7	19	>20	>20	16.5 ± 6.2	>20	>20
3a	6.9 ± 1.6	60 ± 26	2.4 ± 0.2	9	22	>20	9.2 ± 6.3	9.8 ± 4.3	>20	>20
ent-2a	23 ± 13	197 ± 18	2.8 ± 1.0	9	12	>20	4.8 ± 3.1	2.8 ± 1.7	>20	>20
ent-3a	5.7 ± 2.6	501 ± 21	1.6 ± 0.4	88	9.7	>20	>20	11.2 ± 4.8	>20	>20
2b	8.0 ± 2.0	51 ± 16	13 ± 5.0	6	6.3	12.9 ± 7.3	4.3 ± 1.8	2.3 ± 0.9	10.4 ± 0.9	7.0 ± 4.1
3b	7.1 ± 1.8	157 ± 21	7.2 ± 3.9	22	8.5	11.3 ± 5.9	4.9 ± 1.2	6.0 ± 3.8	8.8 ± 2.0	6.8 ± 1.5
2c	8.7 ± 1.2	20 ± 7.0	27 ± 9.0	2	15	–	4.9 ± 4.1	1.6 ± 1.2	3.2	–
3c	23 ± 6.0	334 ± 18	73 ± 6.0	15	18	–	>10	4.5 ± 5.7	>10	–
2d	6.0 ± 0.2	65 ± 7.0	6.4 ± 0.9	11	20	>20	>20	7.6 ± 4.7	>20	14 ± 2.8
3d	1.6 ± 0.1	284 ± 72	2.2 ± 1.1	178	17	>20	>20	10.3 ± 2.9	>20	16 ± 2.8
2e	125 ± 18	705[i]	–	6	–	95 ± 14[j]	103 ± 7.7[j]	46 ± 11[j]	96 ± 11[j]	96 ± 14[j]
3e	6.5 ± 0.7	806[i]	–	124	–	93 ± 10[j]	88 ± 10[j]	46 ± 12[j]	94 ± 6.9[j]	91 ± 9.4[j]
ent-2e	118 ± 5.0	441[i]	–	4	–	67 ± 4.2[j]	88 ± 4.5[j]	33 ± 7.8[j]	77 ± 18[j]	73 ± 9.4[j]
ent-3e	7.5 ± 2.1	1,700[i]	–	227	–	72 ± 1.2[j]	72 ± 33[j]	46 ± 8.6[j]	80 ± 3.3[j]	76 ± 1.9[j]
2f	298 ± 28	4,800[i]	–	162	–	–	–	–	–	–
3f	25%[k]	640[i]	–	–	–	–	–	–	–	–

[a]Guinea pig brain (gp)
[b]Rat liver (rat)
[c]RPMI 8226 cells (hu)
[d]Bladder cancer
[e]Bladder cancer
[f]Small cell lung cancer
[g]Large cell lung cancer
[h]Breast cancer
[i]Result from one measurement. All other results are from three independent experiments
[j]Relative cell growth (%) in relation to an untreated control
[k]Inhibition of radioligand binding at 1 μM concentration of the test compound

The calculated free binding energies of all docked compounds are in good accordance with the experimentally determined receptor binding data. For *ent*-**3a** the calculated ΔG_{bind} is -10.93 ± 0.34 kcal/mol corresponding to a calculated K_i value of 9.7 nM. This K_i value is in good agreement with the K_i values recorded with σ_1 receptors from guinea pig brain ($K_i = 5.7$ nM) and from human RPMI 8226 cell ($K_i = 1.6$ nM) membrane preparations.

The cell growth inhibition potential of compounds **2a-e** and **3a-e** was evaluated in five different cancer cell lines (Table 2). The naphthylmethyl substituted derivatives **2b** and **3b** similarly inhibited the growth and survival of all tested cell lines. The benzyl substituted derivatives **3a** and *ent*-**2a** and the biphenylylmethyl substituted compound **2c** show moderate inhibition of cell growth of the bladder cell line 5637. A clear correlation between σ_1 receptor affinity and growth and survival inhibition could not be determined, however, we did discern a trend revealing sensitivity of A-427 cell line against all tested bicyclic compounds. With the exception of **2b** and **3b**, growth and survival of the other cell lines were not inhibited at compound concentrations up to 10 or 20 μM.

The bridge size does not show additional influence on σ_1 receptor affinity. The K_i values of **2d** and **3d** are in the same range as the K_i values of the ethano-bridged compounds **2a-c** and **3a-c**. Although IC_{50} values are not available for compounds **2e** and **3e**, cell growth of only 33–46 % could be detected for the A-427 cell line, whereas the growth of the other cell lines was not inhibited (Geiger et al. 2007).

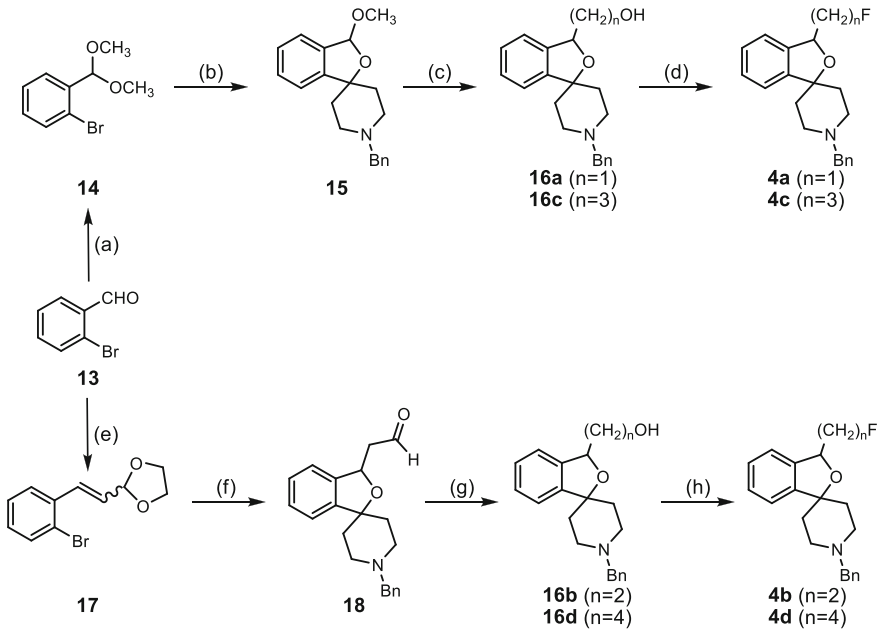
Further experiments directed to elucidate the mechanism of cell growth inhibition showed that *ent*-**2a** induced apoptosis in A-427 cells in a time-dependent manner (Weber et al. 2016).

6 Spirocyclic σ_1 Receptor Ligands

Spirocyclic compounds **4** represent high affinity σ_1 receptor ligands with a favorable pharmacological profile for use as fluorinated PET tracers. The homologous fluoroalkyl derivatives **4a-d** bind σ_1 receptor with K_i values in the low-nanomolar range and show high selectivity over the σ_2 receptor. The (*S*)-configured fluoroethyl substituted compound (*S*)-**4b** is currently investigated as PET tracer for imaging of σ_1 receptors in the CNS of patients suffering from major depression (Fischer et al. 2011; Wang et al. 2013; James et al. 2012). The spirocyclic σ_1 receptor ligands **5** bearing an exocyclic amino moiety allow diverse modifications by the introduction of two *N*-substituents. Furthermore, the existence of cis/trans isomerism increases the diversity of this compound class (Rack et al. 2011).

6.1 Homologous Fluoroalkyl Derivatives

The homologous fluoroalkyl derivatives **4a-d** were developed from the 2-benzofuran **15**, a ligand with high σ_1 receptor affinity ($K_i = 1.1$ nM) and high selectivity over the σ_2 receptor ($K_i = 1{,}280$ nM) and over 60 other receptors and ion



Scheme 3 Synthesis of homologous fluoroalkyl derivatives **4**. Reagents and reaction conditions (**a**) $HC(OCH_3)_3$, p-TosOH, CH_3OH, reflux, 16 h; (**b**) (1) n-BuLi, 1-benzylpiperidin-4-one, THF, −95 °C, 2 h, rt, 4 h; (2) TosOH, CH_3OH, rt, 7 d; (Maier and Wunsch 2002a) (**c**) $n = 1$: (1) trimethylsilyl cyanide, tetracyanoethylene, CH_3CN, reflux, 4 h; (2) H_2SO_4, EtOH, reflux, 7.5 h; (3) $LiAlH_4$, Et_2O, −15 °C, 30 min; (Maier and Wunsch 2002a) $n = 3$: (1) allyltrimethylsilane, $BF_3{\cdot}OEt_2$, CH_2Cl_2, −25 °C, 20 min, 0 °C, 4 h; (2) 9-BBN, THF, rt, 16 h; (3) H_2O_2, NaOH, −25 °C, 45 min, rt 1 h; (Maestrup et al. 2009) (**d**) DAST, CH_2Cl_2, −78 °C to rt., 17 h; (Maestrup et al. 2009) (**e**) $[(CH_2O)_2CHCH_2PPh_3Br]$, K_2CO_3, TDA-1, CH_2Cl_2, reflux, 6 d; (**f**) (1) n-BuLi, THF, 1-benzylpiperidin-4-one, −78 °C, 1 h, rt, 16 h; (2) HCl, THF, rt, 2 h; (**g**) $n = 2$: $NaBH_4$, CH_3CN, 0 °C, 15 min, rt, 16 h; (Maestrup et al. 2011) $n = 4$: (1) ethoxycarbonylmethylentriphenylphosphorane, K_2CO_3, THF, reflux, 23 h; (2) H_2, Pd/C, EtOH, 1 bar, rt, 15 min; (3) $LiAlH_4$, THF, −20 °C, 30 min; (Grosse Maestrup 2010) (h) DAST, CH_2Cl_2, −78 °C to rt, 18–21 h (Maestrup et al. 2011)

channels like the hERG K^+-channel. To eliminate the metabolically unstable acetalic function and to open up the possibility to introduce a fluorine atom into the molecule, a fluoroalkyl residue was installed instead of the acetalic moiety.

The synthesis of fluoroalkyl derivatives **4a-d** started with the acetalization of 2-bromobenzaldehyde **13** to yield the dimethyl acetal **14** (Scheme 3). Homologation of **13** with a Wittig reagent provided the α,β-unsaturated acetal **17**. Halogen-metal-exchange of the acetals **14** and **17** with *n*-BuLi followed by addition of 1-benzylpiperidin-4-one and subsequent transacetalization afforded the spirocyclic 2-benzofurans **15** and **18**. The 2-benzofuranes **15** and **18** served as key intermediates for the synthesis of alcohols **16**. The alcohols were reacted with diethylaminosulfur trifluoride (DAST) to provide the homologous fluoroalkyl

Table 3 σ_1 and σ_2 binding affinities of homologous fluoroalkyl derivatives **4**

	n	σ_1 (gp)[a] K_i [nM] ± SEM	σ_2 (rat)[b] K_i [nM]	σ_1/σ_2 selectivity
4a	1	0.74 ± 0.34	550[c]	743
4b	2	0.59 ± 0.20	785[c]	1,331
4c	3	1.4 ± 0.26	837[c]	620
4d	4	1.2 ± 0.46	489[c]	422
(*S*)-4b	2	2.3 ± 0.2	897[c]	390
(*R*)-4b	2	0.57 ± 0.06	1,650[c]	2,895

[a]Guinea pig brain (gp)
[b]Rat liver (rat)
[c]Result from one measurement. All other results are from three independent experiments, and data presented as mean K_i ± SEM (standard error of the mean)

derivatives **4** (Maestrup et al. 2009, 2011; Grosse Maestrup 2010; Maier and Wunsch 2002a, b).

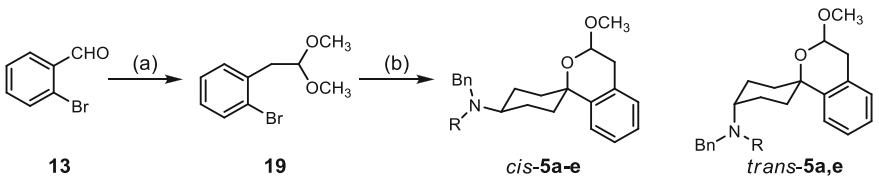
The σ_1 and σ_2 receptor affinity of the homologous fluoroalkyl derivatives **4** was determined with receptor material from guinea pig brain (σ_1) and rat liver (σ_2).

All four fluoroalkyl homologs **4a-d** bind with very high affinity to the σ_1 receptor ($K_i(\sigma_1) = 0.59–1.4$ nM) with high selectivity over the σ_2 subtype (Table 3). The fluoroethyl derivative **4b**, termed fluspidine, shows the most promising ligand-binding profile [K_i (σ_1) = 0.59 nM, K_i (σ_2) = 785 nM)].

All four compounds **4a-d** were also synthesized in their [^{18}F]-labeled form for the use as PET tracers. For radiosynthesis, the alcohols **14** were transformed into the corresponding tosylates. Nucleophilic substitution of the tosylates with K[^{18}F]F complexed with the cryptand Kryptofix 2.2.2 led to the [^{18}F] labeled spirocyclic σ_1 receptor ligands [^{18}F]**4a-d** with high radiochemical purity (>98%) and radiochemical yield (40–50%) with reaction times <30 min (Fischer et al. 2011; Maestrup et al. 2009; Maisonial et al. 2011, 2012). Because of the high target affinity and selectivity, [^{18}F]**4b** was further evaluated in animal studies with female CD-1 mice. [^{18}F]**4b** showed fast and sufficient brain uptake (3.9 and 4.7%ID/g) and high metabolic stability in vivo (>94% parent compound in plasma samples after 30 min, only one metabolite was found). Good concordance between expression of σ_1 receptors and binding site occupancy with [^{18}F]**4b** was found by ex vivo brain section imaging (Fischer et al. 2011). Due to the promising properties of the racemic compound [^{18}F]**4b**, the enantiomers (*R*)- and (*S*)-[^{18}F]**4b** were separated by chiral HPLC of the tosylate **13b** (Holl et al. 2013). The σ_1 receptor affinity was 0.57 nM for (*R*)-[^{18}F]**4b** and 2.3 nM for (*S*)-[^{18}F]**4b**. Thus, the (*R*)-enantiomer is the eutomer.

6.2 Spirocyclic Ligands with Exocyclic Amino Moiety

For the synthesis of spirocyclic ligands with exocyclic amino moiety, the dimethyl acetal **19** was reacted in a bromine lithium exchange to give an aryllithium



13 19 *cis*-**5a-e** *trans*-**5a,e**

Scheme 4 Synthesis of spirocyclic ligands **5** with exocyclic amino moiety. Reagents and reaction conditions (**a**) (1) (methoxymethyl)triphenylphosphonium chloride, KO^{t}Bu, THF, −10 °C, then rt, 16 h; (2) *p*TosOH·H_2O, MeOH, reflux, 72 h; (**b**) (1) *n*-BuLi, THF, −78 °C, 20 min; (2) cyclohexane-1,4-dione, −78 °C, 2 h, rt, 1 h; (3) $CHCl_3$, HCl, rt, 1.5 h; (4) benzylamine, THF, HOAc, $NaBH(OAc)_3$, rt, 2 h; (5) R-CHO, $NaBH(OAc)_3$, CH_2Cl_2, rt, 23 h (Rack et al. 2011)

Table 4 σ_1 and σ_2 binding affinity of spirocyclic ligands **5** with exocyclic amino moiety

	R	σ_1 (gp)[a] K_i [nM] ± SEM	σ_2 (rat)[b] K_i [nM] ± SEM	σ_1/σ_2 selectivity
cis-5a	CH_3	24 ± 4.7	329[c]	14
trans-5a	CH_3	43 ± 18	>1,000	23
cis-5b	C_2H_5	107 ± 25	666 ± 106	6
cis-5c	C_5H_{11}	>1,000	719[c]	–
cis-5d	$PhCH_2$	>1,000	>1,000	–

[a]Guinea pig brain (gp)
[b]Rat liver (rat)
[c]Result from one measurement. All other results are from three independent experiments, and data presented as mean K_i ± SEM (standard error of the mean)

intermediate. After addition to cyclohexane-1,4-dione followed by transacetalization under acidic conditions, reductive amination with benzylamine and $NaBH(OAc)_3$ led to the diastereomeric benzylamines *cis*-**5e** and *trans*-**5e** (R = H, Scheme 4). In order to investigate the influence of the second *N*-substituent, the benzylamines **5e** were transformed into different tertiary amines. Each isomer can adopt different conformations with axially or equatorially oriented amino substituents.

The σ_1 and σ_2 receptor affinity of spirocyclic ligands with exocyclic amino moiety **5** was determined with membrane preparations obtained from guinea pig brain (for σ_1) and rat liver (for σ_2) (Table 4).

The shift of the basic amino group to a position outside of the spirocyclic ring was envisaged to come closer to the required distances between the pharmacophoric elements (benzene ring and amino moiety) according to the models of Glennon and Laggner. The benzylpiperidin **15** (Fig. 6) shows high σ_1 receptor affinity and high selectivity over the σ_2 subtype and over other receptors and ion channels. It was found that small residues at the *N*-atom resulted in low σ_1 affinity whereas a benzyl group turned out to be optimal. The important role of the *N*-benzyl moiety can be explained by the pharmacophore model of Glennon et al. The benzene ring of the annulated pyrane ring interacts with the primary

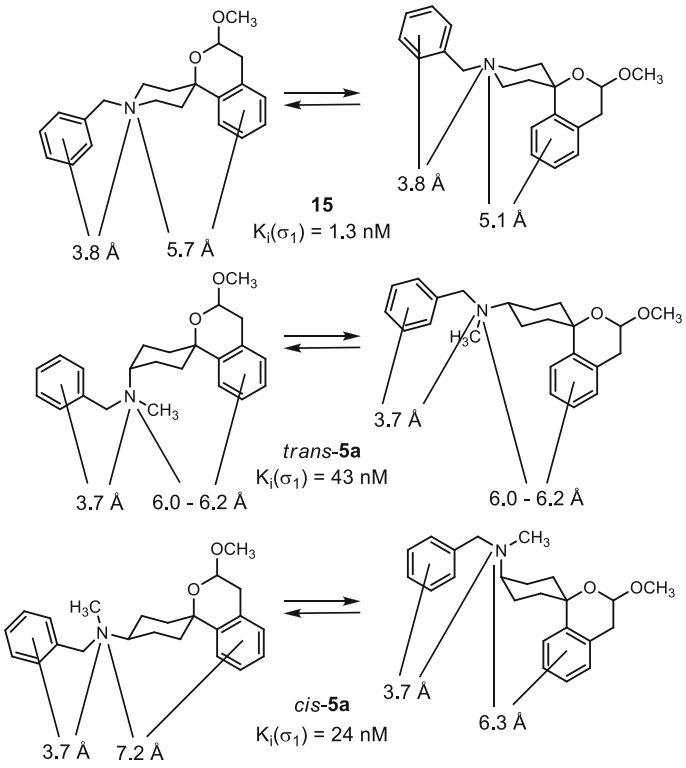


Fig. 6 Distance calculation of spirocyclic σ_1 receptor ligands with endocyclic *N* (**15**) and exocyclic amino moiety (*trans*-**5a** and *cis*-**5a**)

hydrophobic region and the benzyl moiety of the *N*-atom interacts with the secondary hydrophobic region. However, the distance between the *N*-atom and the primary hydrophobic region was too small for both conformers with axially and equatorially oriented phenyl ring. Ideally, the distance should be 6–10 Å due to the pharmacophore model of Glennon et al. In case of **15** the distance was found to be 5.7 and 5.1 Å for the equatorial and axial conformer, respectively (Fig. 6). Therefore it was decided to extend the distance between the *N*-atom and the *O*-heterocycle-annulated benzene ring by exclusion of the *N*-atom from the piperidine ring, resulting in spirocyclic compounds **5** with exocyclic amino group. Another advantage of an exocyclic amino moiety is the possibility to install and to modify two different residues at the *N*-atom.

As a result of the shift of the basic group, the distances between the *N*-atom and the *O*-heterocycle-annulated benzene ring of *cis*-**5a** and *trans*-**5a** are in good concordance with the distances postulated by Glennon et al. However, the decrease in σ_1 affinity ($K_i(\sigma_1) = 24$ and 43 nM) (Rack et al. 2011) provides an example of how receptor binding affinity does not strictly correspond with pharmacophore

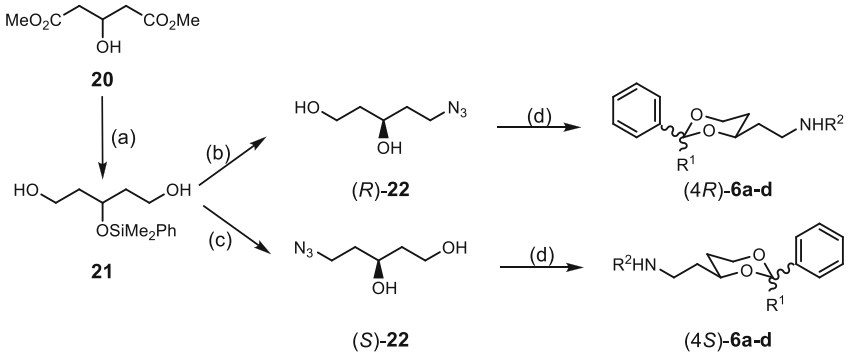
models. Other considerations like entropic factors should be noted. The introduction of the benzylamino moiety leads to an increased flexibility of the *N*-substituent.

The σ_1 receptor affinity of **5** also depends on the second *N*-substituent. Only small groups are tolerated. For small methyl and ethyl groups, the K_i values are 24 and 107 nM, respectively. Bulky residues like pentyl- or benzyl substituents lead to a salient decrease in σ_1 binding affinity ($K_i(\sigma_1) > 1{,}000$ nM). Generally, *cis*-configured diastereomers show higher σ_1 binding affinity than their *trans*-configured diastereomers.

7 1,3-Dioxanes

Racemic 1,3-dioxane **6c** represents a very potent σ_1 receptor antagonist (Utech et al. 2011). With these compounds σ_1 binding affinity depends on the relative configuration of the substituents at the 2- and 4-position, size of the oxygen containing heterocycle, and length of the aminoalkyl side chain. Since the racemic compound **6c**, consisting of a six-membered *O*-heterocycle combined with an aminoethyl side chain, was found to be a promising candidate as σ_1 receptor ligand, the enantiomers were synthesized and their pharmacology evaluated.

For the synthesis of enantiomerically pure 1,3-dioxanes **6**, the enantiomeric azidodiols (*S*)-**22** and (*R*)-**22** were prepared from diester **20** in high enantiomeric excess (Scheme 5). After silylation of **20** and subsequent reduction, the resulting diol **21** was converted into the azidodiols (*S*)-**22** and (*R*)-**22** following two different pathways using lipases as chiral catalysts. Stereoselective acetalization of (*S*)-**22** and (*R*)-**22** with benzaldehyde or acetophenone led to enantiomerically pure azido-



Scheme 5 Synthesis of enantiomerically pure 1,3-dioxanes **6**. Reagents and reaction conditions. (**a**) (1) $Me_2PhSiCl$, imidazole, CH_2Cl_2; (2) $LiBH_4$, Et_2O; (**b**) (1) IPA, lipase *Candida Antarctica* B, MTBE; (2) lipase *Burkholderia cepacia*, $NaHCO_3$; (3) $Zn(N_3)_2{\cdot}(pyridine)_2$, DIAD, PPh_3, toluene; (4) K_2CO_3, CH_3OH; (5) HCl; (**c**) (1) IPA, lipase *Burkholderia cepacia*, MTBE; (2) $Zn(N_3)_2{\cdot}(pyridine)_2$, DIAD, PPh_3, toluene; (3) K_2CO_3, CH_3OH; (4) HCl; (Kohler and Wunsch 2006, 2012) (**d**) (1) Ph-C(=O)R^1, pTosOH, toluene, Dean Stark apparatus, 4 h; (2) H_2, Pd/C, rt, 5 h; $R^2 = PhCH_2$: (3) benzaldehyde, $NaBH(OAc)_3$, CH_2Cl_2, rt, 16 h (Kohler et al. 2012). *IPA* isopropenyl acetate, *MTBE* methyl *tert*-butyl ether, *DIAD* diisopropyl azodicarboxylate

Table 5 σ_1 and NMDA receptor binding affinities of 1,3-dioxanes **6a-d**

	R^1	R^2	σ_1 (gp)[a] K_i [nM] ± SEM	NMDA (p)[b] K_i [nM] ± SEM
(*S*,*R*)-6a	H	H	>10,000	>10,000
(*R*,*S*)-6a	H	H	>10,000	>10,000
(*R*,*R*)-6b	CH_3	H	>10,000	46 ± 17
(*S*,*S*)-6b	CH_3	H	>10,000	6,120 ± 630
(*S*,*R*)-6c	H	$PhCH_2$	6.0 ± 1.0	>10,000
(*R*,*S*)-6c	H	$PhCH_2$	50 ± 19	>10,000
(*R*,*R*)-6d	CH_3	$PhCH_2$	17 ± 2	>10,000
(*S*,*S*)-6d	CH_3	$PhCH_2$	11 ± 3	>10,000

[a]Guinea pig brain (gp)
[b]Pig brain cortex (p)

1,3-dioxanes, which were subsequently reduced with H_2 and Pd/C to obtain the primary amines **6a** and **6b**. Further functionalization of the amino moiety was performed by reductive monobenzylation with benzaldehyde and $NaB(OAc)_3$ to yield the benzylamines **6c** and **6d**.

The σ_1 and σ_2 receptor affinity of 1,3-dioxanes **6** was determined with tissue membrane preparations from guinea pig brain (for σ_1) and rat liver (for σ_2). The 1,3-dioxanes **6** were also tested against the PCP binding site of the NMDA receptor using pig brain cortex membrane preparations (Table 5).

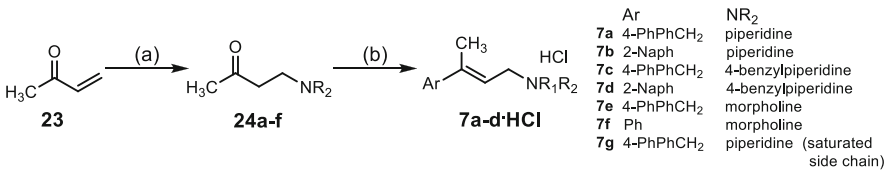
We found that both enantiomers of the primary amines **6a** and **6b** do not bind σ_1 ($K_i(\sigma_1) > 10{,}000$ nM) in this assay. According to the pharmacophore model of Glennon et al. (Glennon 2005; Glennon et al. 1994) affinity for σ_1 should increase by introducing an *N*-benzyl group as a second hydrophobic residue flanking the basic amino moiety as it is shown for the secondary amines **6c** and **6d**. In the case of benzylamines the orientation of the phenyl ring at the 1,3-dioxane ring has minimal influence on σ_1 affinity as **6a** and **6b** show comparable K_i values of 6.0 and 17 nM for the (4*R*)-configured enantiomers and 50 and 11 nM for the (4*S*)-configured enantiomers. Compound (*S*,*R*)-**6c** with equatorially oriented 2-phenyl moiety shows high σ_1 affinity ($K_i(\sigma_1) = 6.0$ nM).

Regarding σ_1/σ_2 selectivity, we found that primary amines **6a** and **6b** do not bind σ_1 and the benzyl amines **6c** and **6d** bind σ_2 with low affinity ($K_i(\sigma_2) > 200$ nM).

Depending on the absolute configuration the primary amines **6b** with axially oriented phenyl moiety reveal high affinity to the PCP binding site of the NMDA receptor with K_i values of 46 nM ((*R*,*R*)-**6b**) but only 6,120 nM for (*S*,*S*)-**6b**, respectively. The equatorial orientation of the phenyl ring (**6a**) as well as the introduction of a benzyl group at the amino moiety (**6c** and **6d**) led to complete loss of NMDA affinity (K_i(NMDA) > 10,000 nM).

The benzyl substituted 1,3-dioxane (*S*,*R*)-**6c** represents the most potent candidate among the secondary amines with high σ_1 affinity ($K_i(\sigma_1) = 6.0$ nM) and high selectivity over the σ_2 subtype and the NMDA receptor.

In further studies performed with racemic **6c** ($K_i(\sigma_1) = 19$ nM), promising results were obtained in a capsaicin-induced pain assay with mice. In these studies,



Scheme 6 Synthesis of arylalkenylamines **7a-f**. Reagents and reaction conditions (**a**) HNR_2, PEG 400, rt; (**b**) (1) Ar-Br, t-BuLi, Et_2O, −78 °C to rt; (2) 37% HCl, rt; (3) 1 M NaOH; (4) 37% HCl, rt; (5) crystallization from acetone (Rossi et al. 2011). *Naph* naphthyl

Table 6 σ_1 and σ_2 affinity of arylalkenylamines **7a-g**

	σ_1 (gp)[a] K_i [nM] ± SEM	σ_2 (rat)[b] K_i [nM] ± SEM	σ_1/ σ_2 selectivity	clog*P*	clogD	MW
7a	0.86 ± 0.4	111 ± 21	129	5.32	3.82	291.43
7b	0.97 ± 0.3	35 ± 9	36	4.66	3.07	265.39
7c	7.0 ± 0.9	18 ± 1.7	3	7.19	5.60	381.55
7d	23 ± 2.6	16 ± 1.1	1	6.53	4.86	355.52
7e	12 ± 2.0	386 ± 27	33	4.25	4.17	293.40
7f	>1,000	>1,000	–	2.61	2.51	217.31
7 g	0.70 ± 0.3	103 ± 10	147	5.43	3.00	293.45

[a]Guinea pig brain
[b]Rat liver

even a very low dose of 0.25 mg/kg, *rac*-**6c** has high anti-allodynic activity (Utech et al. 2011).

8 Arylalkenylamines

Arylpropenylamines of type **7** show high σ_1 binding affinity and high σ_1/σ_2 selectivity. The influence of the novel σ_1 ligands on nerve growth factor (NGF)-induced neurite outgrowth was evaluated in the in vitro PC12 cell neurite sprouting assay.

Michael addition of cylic amines to unsaturated ketone **23** led to the β-aminoketones **24**. Subsequent nucleophilic aryllithium addition followed by dehydration with HCl provided the arylalkenylamines **7**, which were crystallized as HCl salts (*E*)-**7a-f** (Scheme 6). The racemic arylalkylamine **7g** was obtained by catalytic hydrogenation of **7a**.

The σ_1 and σ_2 receptor binding affinity of compounds **7a-g**·HCl was tested using guinea pig brain (for σ_1) and rat liver (for σ_2) membrane preparations. Additionally, the selectivity towards the PCP binding site of the NMDA receptor and against μ- and κ-opioid receptors was determined (Table 6).

Piperidinyl substituted compounds **7a** and **7b** reveal high σ_1 receptor affinity independent of the aromatic residue ($K_i(\sigma_1)$ = 0.86 and 0.97 nM). Interestingly, naphthalen-2-yl- or biphenyl-4-yl residues appear to be important for high σ_1

binding affinity of morpholinyl substituted compounds since only low σ_1 affinity is found when a phenyl substituent is present as aromatic moiety (**7f**, $K_i(\sigma_1)$ >1,000 nM). The tested compounds show high selectivity over the σ_2 receptor subtype, opioid receptors, and NMDA receptors (the PCP binding site). High σ_2 binding affinity was found only for 4-benzylpiperidinyl substituted derivatives **7c** and **7d,** with K_i values of 18 and 16 nM, respectively. **7a** represents the most potent and selective σ_1 receptor ligand of this set of compounds ($K_i(\sigma_1) = 0.86$ nM, $\sigma_1/\sigma_2 = 129$). Therefore the corresponding arylalkylamine **7g** was included in this study. Receptor binding studies revealed similar σ_1 binding affinity ($K_i(\sigma_1) = 0.70$ nM) and selectivity ($\sigma_1/\sigma_2 = 147$) (Rossi et al. 2011).

clog*P* and clog*D* values were calculated for **7a-g.** Their drug-like properties were confirmed according to Lipinski's "rule of five." With the exception of **7c** (log*D* > 5) all compounds fulfill the "rule of five," i.e., clogD <5, molecular weight < 500, H-bond acceptors <10, and H-bond donors <5.

In order to determine whether the top compounds **7a** and **7g** function as agonists or antagonists their influence on nerve growth factor (NGF)-induced neurite outgrowth in PC12 cells was evaluated. **7g** potentiated the neurite outgrowth at lower concentrations, consistent with agonist activity. This effect was blocked by co-treatment with the σ_1 receptor antagonist BD-1063, demonstrating the participation of σ_1 receptors. In contrast, (*E*)-**7a** did not significantly increase neurite sprouting (Rossi et al. 2011).

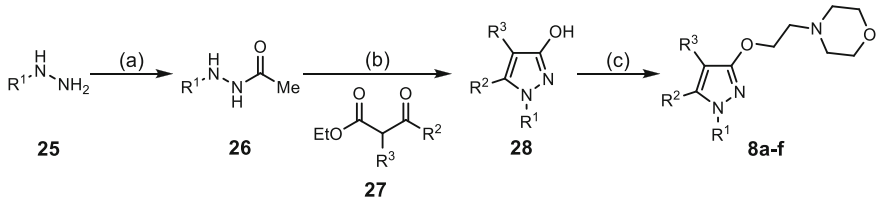
9 Morpholinoethoxypyrazoles

Substituted 1-arylpyrazoles with a basic amino function represent a promising class of σ_1 receptor antagonists. For high σ_1 binding affinity, the distance between the basic amino moiety and the pyrazole ring is of major importance. In previous studies an ethylenoxy spacer and a morpholino residue as the *N*-component resulted in high σ_1 affinity and excellent selectivity over the σ_2 subtype.

For the synthesis of morpholinoethoxypyrazoles **8a-f**, the 3-hydroxypyrazole **28** was prepared in a two-step reaction sequence starting from arylhydrazines **25**. At first the terminal amino group of hydrazines **25** was protected by acetylation (Scheme 7). Reaction of **26** with β-ketoesters **27** led to the 3-hydroxypyrazoles **28** with high regioselectivity. Subsequent reaction with 4-(2-chloroethyl)morpholine provided the morpholinoethoxypyrazoles **8a-f** (Diaz et al. 2012).

The σ_1 and σ_2 binding affinity of morpholinoethoxypyrazoles **8a-f** was determined with human σ_1 receptors (from transfected HEK-293 cell membrane preparations) and membrane preparations from guinea pig brain (for σ_2).

Generally, the morpholinoethoxypyrazoles **8a-f** show only very low affinity for the σ_2 receptor. Affinity for σ_1 depends on the substitution pattern of the pyrazole ring. Substitution at position 1 with aromatic residues (**8b-f**) produces high σ_1 binding affinity. Non-aromatic residues (*tert*-butyl, **8a**) produce a salient decrease in σ_1 binding affinity (Table 7). Only small residues (e.g., CH_3, H) are tolerated at 4- and 5-position of the pyrazole ring. In the naphthyl series even a methyl group in



Scheme 7 Synthesis of morpholinoethoxypyrazoles **8a-f**. Reagents and reaction conditions (**a**) Ac_2O, toluene, rt; (**b**) PCl_3, 50 °C, 2 h; NaH, DMF, 60 °C, 4 h; (**c**) 4-(2-chloroethyl)morpholine, K_2CO_3, NaI, DMF, 95 °C, 18 h (Diaz et al. 2012)

Table 7 σ_1 and σ_2 binding affinity of morpholinoethoxypyrazoles **8a-f**

	R^1	R^2	R^3	σ_1 (h)[a] K_i [nM] ± SEM	σ_2 (gp)[b] K_i [nM] ± SEM	σ_1/ σ_2 selectivity
8a	*tert*-butyl	H	H	>1,000	>1,000	–
8b	4-Chlorophenyl	H	H	18 ± 1.5	357 ± 357	20
8c	2-Naph	CH_3	H	17 ± 7.0	>1,000	–
8d	2-Naph	CH_3	CH_3	139 ± 9	>1,000	–
8e	3,4-Dichlorophenyl	CH_3	CH_3	9.4 ± 1.8	351 ± 400	37
8f	3,4-Dichlorophenyl	CH_3	C(=O) CH_3	741 ± 134	>1,000	–

[a]Human σ_1 receptor from transfected HEK-293 cell membrane preparations (h)
[b]Guinea pig brain membrane preparations (gp)

position 4 seems to be detrimental for high σ_1 affinity (**8d**, $K_i(\sigma_1) = 139$ nM). The introduction of larger moieties in position 4 (e.g., **8f**, C(=O)Me) produces a salient decrease in σ_1 binding affinity ($K_i(\sigma_1) = 741$ nM). The 5-alkoxy regioisomer of the most promising ligand **8c** ($K_i(\sigma_1) = 17$ nM) shows a complete loss of σ_1 affinity ($K_i > 1{,}000$ nM) (Diaz et al. 2012).

The high σ_1 binding affinity of naphthylpyrazole **8c** (also known as S1RA and E-52862) cannot be explained completely by the common pharmacophore models. The 2-[1-(2-naphthyl)pyrazol-3-yloxy]ethyl moiety fits well into the primary hydrophobic region of the Glennon model, tolerating sterically demanding residues. However, the morpholine ring does not fulfill the requirements to address the second hydrophobic region.

Ligands **8** with excellent σ_1 receptor binding affinity and selectivity were further evaluated for their activity at the hERG channel and for efficacy in mouse models of neuropathic pain. Naphthylpyrazole **8c** proved to be the most promising candidate with regard to metabolic stability, interaction with the hERG channel ($IC_{50} > 10$ μM), and analgesic activity in different pain models (Diaz et al. 2012). It was found that **8c** shows dose-dependent analgesic effects in both the capsaicin-induced hypersensitivity and the formalin-induced pain model. In the partial sciatic nerve ligation mouse model, **8c** shows dose-dependent inhibition of

thermal hypersensitivity and mechanical allodynia, comparable to the effects of pregabalin, the gold-standard for the treatment of neuropathic pain.

The selectivity of **8c** towards 170 other targets including various receptors and ion channels was shown. With the exception of moderate affinity for the human serotonin 5-HT_{2B} receptor (K_i(5-HT_{2B}) = 328 nM) other targets were not engaged by **8c**. The antagonist profile of **8c** was verified using phenytoin as an allosteric modulator of σ_1.

The chemical properties of **8c** meet Lipinski's "rule of five." The pharmacokinetic properties were evaluated in mice. Due to the acceptable solubility and high metabolic stability, a good oral bioavailability can be assumed.

In light of all of the aforementioned properties, **8c** entered clinical trials. Passing the single and multiple dose phase I clinical study provided proof-of-concept that **8c** is safe and well tolerated by healthy humans. Thus, the development of **8c** will be continued into phase II clinical trials (Abadias et al. 2013).

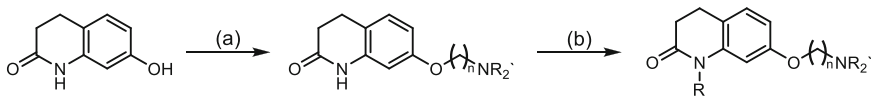
10 3,4-Dihydro-2(1*H*)-quinolones

3,4-Dihydro-2(1*H*)-quinolones **9** were developed following the idea of combining a piperidine or morpholine basic element (as realized in the σ_1 receptor antagonist **8c**) with the quinolone scaffold, which was identified as the interacting element at the σ_1 receptor (Oshiro et al. 2000). The aim was to obtain compounds with high affinity for the σ_1 receptor and potent anti-nociceptive properties as shown for S1RA.

For the synthesis of quinolones **9**, 7-hydroxyquinolone **29** was alkylated with dibromoalkans with various length of the alkyl group (Scheme 8). Subsequent reaction with morpholine or piperidine led to the amines **30**, which were converted into 1-alkylated quinolones **9** by reaction with benzyl bromides or iodomethane in presence of NaH (Lan et al. 2014).

The σ_1 and σ_2 affinity of quinolones **9** was determined in competition experiments using guinea pig brain membrane preparations (Table 8).

The distance between the quinolone scaffold and the morpholine ring has a strong impact on σ_1 binding affinity. Whereas **9a** with an ethylene spacer has negligible affinity for σ_1 ($K_i(\sigma_1) > 2{,}000$ nM), the corresponding homolog **9b** bearing three CH_2 moieties in the side chain binds σ_1 with high affinity ($K_i(\sigma_1) = 14.8$ nM). Elongation by introduction of additional methylene moieties



Scheme 8 Synthesis of quinolones **9**. Reagents and reaction conditions (**a**) (1) $Br(CH_2)_nBr$, K_2CO_3, acetone, reflux; (2) HNR_2, K_2CO_3, KI, CH_3CN, reflux; (**b**) NaH, DMF or THF, 0–50 °C (Lan et al. 2014)

Table 8 σ_1 and σ_2 binding affinity of 3,4-dihydro-2(1*H*)-quinolinones **9**

	R^1	*n*	NR_2'	σ_1 (gp)[a] K_i [nM] ± SEM	σ_2 (gp)[a] K_i [nM] ± SEM	σ_1/ σ_2 selectivity
9a	$PhCH_2$	2	Morpholine	>2,000	>2,000	–
9b	$PhCH_2$	3	Morpholine	14.8 ± 0.8	471 ± 38	32
9c	H	3	Piperidine	89 ± 14	288 ± 52	3
9d	CH_3	3	Piperidine	34 ± 3	357 ± 131	10
9e	$PhCH_2$	3	Piperidine	1.84 ± 0.33	662 ± 42	360
9f	4-F-Ph-CH_2	3	Piperidine	1.22 ± 0.45	1,301 ± 204	1,066
9 g	$PhCH_2$	4	Morpholine	60 ± 4.4	530 ± 46	9
9 h	$PhCH_2$	5	Morpholine	88 ± 5.1	1,811 ± 37	20
9i	$PhCH_2$	6	Morpholine	682 ± 31	2,000	–

[a]Guinea pig brain (gp)

decreased σ_1 affinity in the order $n = 3 > n = 4 > n = 5 > n = 6$. Compound **9i**, with a hexamethylene linker, had the lowest σ_1 binding affinity of this set of compounds with a K_i value of 682 nM. Replacement of the morpholine ring of the most potent ligand **9b** of this series by a piperidine ring led to an almost tenfold increase in σ_1 binding affinity ($K_i(\sigma_1) = 1.84$ nM). For the promising piperidine derivatives, the effect of the quinolinone *N*-substituent on σ_1 receptor binding was evaluated by synthesizing different substituted analogs **9c**, **9d,** and **9f**. It has been found that small residues led to decreased σ_1 affinity compared with the *N*-benzyl substituted derivative **9e** ($K_i(\sigma_1) = 1.84$ nM). In the case of substitution with a proton or a methyl group, the K_i values were only 89 nM (**9c**) or 34 nM (**9d**). The substitution of the phenyl ring with an electron-withdrawing fluorine atom led to a slight increase in σ_1 binding affinity (**9f**, $K_i(\sigma_1) = 1.22$ nM).

Regarding σ_1/σ_2 selectivity, it was found that the most potent σ_1 ligand **9f** has also the highest selectivity with a σ_2 $K_i > 1{,}000$ nM. For the piperidine derivatives **9c-f**, the σ_2 affinity increased with decreased size of substituents. The secondary lactam **9c** shows the highest σ_2 affinity and the lowest σ_1/σ_2 selectivity of this set of compounds ($K_i(\sigma_2 = 288$ nM, $\sigma_1/\sigma_2 = 3$). The chain length between the quinolone scaffold and the morpholine residue also influences affinity for the σ_2 receptor. Compounds **9a** ($n = 2$), **9h** ($n = 5$), and **9i** ($n = 6$) do not show σ_2 affinity. However, compounds **9b** and **9g** with a trimethylene or tetramethylene linker displayed moderate σ_2 affinity, with a K_i of approximately 500 nM.

The most potent ligand **9f** was further evaluated for its anti-nociceptive activity in the formalin-induced pain assay. It was found that **9f** dose-dependently reduced both phases of the pain response with ED_{50} values of 49.4 ± 4.1 and 50.5 ± 2.5 mg/kg for the acute phase I and the longer-lasting tonic phase II, respectively. The σ_1 antagonist activity of **9e** was shown using phenytoin as an allosteric modulator of σ_1.

11 Conclusion

During the past several years, the fields of σ_1 receptor chemistry and pharmacology have made remarkable progress. Various pharmacophore models of σ_1 ligands, a 3D homology model of the σ_1 receptor, its structure in solution (NMR), and its structure in the solid state (X-ray crystallography) have been reported, allowing a closer look at the binding properties of σ_1 receptors to their ligands. Evidence of σ_1 as a promising target for the development of new therapeutic approaches has been demonstrated. The σ_1 antagonist S1RA (**8c**) is currently in clinical trials for the treatment of neuropathic pain. Bicyclic piperazines **2** and **3** inhibit the growth of small cell lung cancer cells (A-427 cells) in a dose-dependent manner, demonstrating their potential as new tumor therapeutics. The (*S*)-configured spirocyclic σ_1 antagonist **4b** (fluspidine) with a fluoroethyl side chain has been developed as tracer for positron emission tomography (PET) and is currently in clinical trials for imaging and analysis of the brain of patients suffering from major depression.

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Sigma-1 (σ_1) Receptor in Memory and Neurodegenerative Diseases

Tangui Maurice and Nino Goguadze

Contents

Abstract

The sigma-1 (σ_1) receptor has been associated with regulation of intracellular Ca^{2+} homeostasis, several cellular signaling pathways, and inter-organelle communication, in part through its chaperone activity. In vivo, agonists of the σ_1 receptor enhance brain plasticity, with particularly well-described impact on learning and memory. Under pathological conditions, σ_1 receptor agonists can induce cytoprotective responses. These protective responses comprise various complementary pathways that appear to be differentially engaged

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according to pathological mechanism. Recent studies have highlighted the efficacy of drugs that act through the σ_1 receptor to mitigate symptoms associated with neurodegenerative disorders with distinct mechanisms of pathogenesis. Here, we will review genetic and pharmacological evidence of σ_1 receptor engagement in learning and memory disorders, cognitive impairment, and neurodegenerative diseases, including Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, multiple sclerosis, and Huntington's disease.

Keywords
Alzheimer's disease • Amyotrophic lateral sclerosis • Huntington's disease • Learning and memory • Multiple sclerosis • Neuroprotection • Parkinson's disease • σ_1 polymorphisms • σ_1 receptor

1 The Sigma-1 (σ_1) Receptor in Cellular Physiology

The sigma-1 (σ_1) receptor was initially thought to be a subtype of opioid receptors (Martin et al. 1976). It is now clearly defined as a unique membrane-associated protein (Hanner et al. 1996; Schmidt et al. 2016) with chaperone activity (Hayashi and Su 2007). It is expressed in tissue throughout the body including central nervous system (CNS) cells, in neurons, astrocytes, oligodendrocytes, and microglia (Alonso et al. 2000). Its amino acid sequence predicts a 26 kDa protein (Hanner et al. 1996; Schmidt et al. 2016). It is expressed primarily in the intracellular endomembrane networks where it associates with the glucose-related protein 78/binding immunoglobulin protein (GRP78/BiP) (Hayashi and Su 2007). It has been reported to be enriched at mitochondria-associated endoplasmic reticulum (ER) membranes (MAM) (Hayashi and Su 2007) where it regulates interorganelle calcium exchange. It also has been reported to be expressed at the plasma membrane where it associates with ceramide-enriched lipid rafts microdomains (Hayashi and Su 2007, 2003, 2004, 2005, 2010). The σ_1 receptor acts as a chaperone, binding several client proteins.

It can be activated or inactivated by numerous pharmacological compounds, including psychostimulants, antipsychotics, opioids, muscarinic receptor ligands, D2 dopamine receptor ligands, N-methyl-D-aspartate (NMDA) receptor ligands, monoamine transporters inhibitors, serotonin reuptake inhibitors, monoamine oxidase inhibitors, steroids, some peptides like neuropeptide Y, and calcitonin gene-related peptide [reviewed in (Maurice and Su 2009)]. Under physiological conditions, the σ_1 receptor is thought to be associated with the ER-resident chaperone BiP at MAM (Hayashi and Su 2007). Under acute cellular stress or in response to treatment with agonists, the σ_1 receptor dissociates from BiP and binds alternate client or partner proteins including the inositol 1,4,5-trisphosphate (IP_3) receptor, thus enhancing calcium entry into the mitochondria (Hayashi et al. 2000; Hayashi and Su 2007). Ca^{2+} entry into mitochondria promotes redox reactions and ATP production, thereby regulating Ca^{2+}-dependent enzymes in the tricarboxylic acid cycle (Rizzuto et al. 2004). Recently, an alternative mechanism of Ca^{2+} modulation

by the σ_1 receptor has been proposed. Brailoiu et al. (2016) described that the psychostimulant drug cocaine inhibits store-operated Ca^{2+} entry (SOCE), a Ca^{2+} influx mechanism promoted by depletion of intracellular Ca^{2+} stores, in rat brain microvascular endothelial cells through a σ_1 receptor-dependent mechanism. Cocaine-induced SOCE inhibition was blocked by shRNA knockdown of the σ_1 receptor or the σ_1 receptor antagonists BD1063 and NE100. The σ_1 receptor therefore may regulate Ca^{2+} homeostasis by various intracellular signal transduction pathways involving protein–protein interactions. The σ_1 receptor appears to constitute a unique class of protein that influences a range of cellular systems. It has been shown that ectopic overexpression of σ_1 receptor or treatment with σ_1 receptor agonists can counteract ER stress, whereas decreasing its expression enhances apoptosis (Hayashi and Su 2007). In addition, after activation, σ_1 receptor can translocate to the plasma membrane or other cell compartments and bind to various receptors and membrane-associated proteins, including ion channels, kinases, G-protein coupled receptors, or trophic factor receptors (Martina et al. 2007; Navarro et al. 2010, 2013; Kourrich et al. 2013).

Among other effects with evident physiological impacts on brain plasticity and memory, activation of the σ_1 receptor modulates voltage-gated ion channels involved in the initiation and shaping of action potentials (Soriani et al. 1999; Zhang and Cuevas 2002), NMDA-induced neuronal firing in the hippocampus (Monnet et al. 1990; Martina et al. 2007), and recruitment and coupling of Ca^{2+}-dependent nitric oxide synthase (NOS) to postsynaptic density protein-95 (PSD95) (Cao et al. 2005; Yang et al. 2010). The σ_1 receptor is also able to shape cellular plasticity in neuronal cells by directly modulating the activity of pleiotropic transcription factors such as nuclear factor κB (NFκB), cyclic adenosine monophosphate (cAMP) response element-binding (CREB) protein, and c-fos. These transcription factors are involved in the modulation of pro- and anti-inflammatory genes as well as cell death and survival (Meunier and Hayashi 2010). At the plasma membrane, the σ_1 receptor may directly control dendritic spine arborization by increasing Rac-GTP, in part by regulating levels of intracellular reactive oxygen species (ROS) (Tsai et al. 2009). A direct interaction between the σ_1 receptor and Rac1-GTPase was described in brain mitochondria (Natsvlishvili et al. 2015). The σ_1 receptor therefore constitutes a unique class of proteins influencing and participating in a wide range of biological pathways, including Ca^{2+} signaling at the ER and controlling several families of ion channels at the plasma membrane and MAM. The σ_1 receptor helps to maintain ER-mitochondria exchanges and trigger transcription factor expression. The σ_1 receptor-mediated neuromodulation, affecting several cellular pathways, has an important role on brain plasticity either in physiological conditions, particularly learning and memory processes, or on brain preservation, particularly during neurodegenerative insults.

2 Role of σ_1 Receptors in Learning and Memory

Agonists of the σ_1 receptor are effective anti-amnesic compounds. This has been demonstrated in a number of pharmacological and pathological models of learning and memory impairment in rodents. In particular, new σ_1 agonists are routinely validated in vivo against scopolamine-induced learning deficits, a model of muscarinic acetylcholine receptor (mAChR) blockade. For instance, the σ_1 receptor agonist LS-1-137, an *N*-(1-benzylpiperidin-4-yl)phenylacetamide analog (Malik et al. 2015), the σ_1 receptor agonist (4R,5S)-2-(5-methyl-2-oxo-4-phenyl-pyrrolidin-1-yl)-acetamide [E1R; (Zvejniece et al. 2014)], or the mixed mAChR/σ_1 receptor agonists ANAVEX1-41 or ANAVEX2-73, two diphenyl-3-furanmethanamine derivatives (Espallergues et al. 2007; Villard et al. 2009) have recently been characterized as anti-amnesic drugs against scopolamine-induced learning impairment. The efficacy of σ_1 receptor agonists as symptomatic drugs in cognition has been described not only in cholinergic amnesia models (e.g., scopolamine, mecamylamine, *p*-chloroamphetamine, forebrain lesions) but also in glutamatergic models of learning deficit. Learning impairment induced by the noncompetitive NMDA receptor antagonist dizocilpine (MK-081) has been used to demonstrate that the positive modulation exerted by the σ_1 receptor on NMDA neurotransmission, suggested in vitro (Monnet et al. 1992b, 1995) and in vivo using extracellular recordings of the NMDA-induced firing of pyramidal neurons in the CA3 hippocampal area (Monnet et al. 1990, 1992a), has behavioral consequences. The efficacy of σ_1 receptor agonists in alleviating dizocilpine-induced learning impairment also points to the potential utility of these drugs in treating schizophrenia-related cognitive deficits, in particular since hypoglutamatergy models have been considered as highly pertinent for mimicking the negative symptoms of schizophrenia (Meltzer et al. 2013). Interestingly, σ_1 receptor ligands tested in both the scopolamine and dizocilpine models showed a similar active dose-range in vivo (Villard et al. 2009, 2011). Activation of the σ_1 receptor therefore appeared to similarly modulate the activity of the two neurotransmission systems involved in memory processes in the limbic and cortical structures, namely the cholinergic and glutamatergic systems.

The cholinergic system is crucial for learning, consolidation, and retrieval phases of the memory processes. Cholinergic basal forebrain neurons in the *nucleus basalis magnocellularis* innervate the cerebral cortex, amygdaloid complex, and hippocampus, all structures involved in memory formation (Aigner 1995). Activation of σ_1 receptors by agonists provokes ACh release. This has been shown both in vitro and in vivo. (+)-SKF-10,047, igmesine, and cutamesine (SA4503) potentiate KCl-induced release of [^{3}H]-ACh from rat hippocampal slices (Junien et al. 1991; Horan et al. 2002). (+)-SKF-10,047, (+)-3-PPP, (+)-pentazocine, DTG, and cutamesine acutely and dose-dependently increase extracellular ACh levels in the frontal cortex and hippocampus as measured by in vivo microdialysis in freely moving rats (Matsuno et al. 1993, 1995). The mechanism by which these σ_1 receptor ligands induce ACh release involves Ca^{2+} mobilization through IP_3 receptor and voltage-gated K^+ and Ca^{2+} channels (Hayashi et al. 2000; Foskett et al. 2007).

Some σ_1 receptor agonists enhance NMDA-induced firing in the hippocampus at very low doses (Monnet et al. 1990, 1992a, b, 1995). Neuroactive steroids with affinity for σ_1 receptors such as dehydroepiandrosterone (DHEA) sulfate or pregnenolone sulfate enhanced paired-pulse facilitation or facilitate induction of frequency-dependent long-term potentiation (LTP) in rat hippocampal CA1 pyramidal cells (Schiess and Partridge 2005; Chen et al. 2006). The latter effect was proposed to involve Src-dependent NMDA receptor signaling and regulation of the tyrosine phosphorylation of NMDA receptor subunit 2B (NR2B). Tyrosine phosphorylation of NR2B decreased after reversible forebrain ischemia in rats and improved after repetitive administration of DHEA sulfate, whereas NR1 remained unchanged (Li et al. 2006). Moreover, (+)-SKF-10,047, PRE-084, and (+)-pentazocine increased the expression of NR2A and NR2B, as well as PSD95, in the rat hippocampus (Pabba et al. 2014). Treatment with σ_1 receptor agonists leads to increased interaction between NR2 subunits and σ_1 receptors and promotes trafficking of NMDA receptors to the cell surface. The σ_1 receptor interacts with NMDA receptors through the regulation of a small conductance Ca^{2+}-activated K^+ current (SK channels). Using patch-clamp whole-cell recordings in CA1 pyramidal cells of rat hippocampus, Martina et al. (2007) described that (+)-pentazocine potentiated NMDA receptor responses and LTP by preventing the opening of SK channels, a channel known to shunt NMDA receptor responses. These electrophysiological parameters were examined in σ_1 receptor knockout (*SIGMAR1* KO) mice. Using whole-cell patch-clamp recordings from CA1 pyramidal neurons, Snyder et al. (2016) observed no change in action potential or basic cellular characteristics and no change in presynaptic function, as indicated by a similar paired-pulse ratio and miniature excitatory postsynaptic current frequency. The AMPA and NMDA receptors were unaffected, with no difference in AMPA/NMDA ratio or decay kinetics, in *SIGMAR1* KO compared to wild-type mice (Snyder et al. 2016). However, a small but significant reduction in the magnitude of LTP was measured, suggesting that basic cellular physiology is unaffected after σ_1 receptor ablation, but the neuronal network is partially compromised. At the behavioral level, young male *SIGMAR1* KO mice, at 2 months of age, showed signs of anxiety in procedures including the open-field, passive avoidance and elevated plus-maze, and an enhanced response to stress in the forced swim test (Chevallier et al. 2011). In male animals, the σ_1 receptor ablation therefore increased stress and anxiety responses but memory responses were unchanged. However, female *SIGMAR1* KO mice showed memory alterations in spontaneous alternation and water-maze learning paradigms, and this phenotype increased with age. Of note, both 2- and 14-month old female *SIGMAR1* KO mice showed decreased plasma levels of 17β-estradiol and a supplementation treatment with the hormone reversed the memory deficits in young and aged mice (Chevallier et al. 2011). This suggested that σ_1 receptor ablation has a developmental impact on the steroidal tonus.

Agonists of the σ_1 receptor are promising symptomatic drugs in rodent models of cognitive alterations related to pathological aging and neurodegenerative diseases. First, igmesine and PRE-084, in the low mg/kg dose-range, improved learning ability in the senescence-accelerated mouse SAMP/8 (Maurice et al. 1996). Second,

these compounds also alleviated the memory deficits induced by amyloid toxicity in pharmacological models of Alzheimer's disease (AD). (+)-pentazocine, PRE-084, cutamesine, dimemorphan, ANAVEX1-41, ANAVEX2-73, and σ_1 receptor binding neuroactive steroids attenuated learning deficits in mice that received a direct intracerebroventricular injection of oligomerized $A\beta_{25\text{-}35}$ peptide, which produces neurotoxicity closely related to AD pathology (Maurice et al. 1996; Zussy et al. 2011). All σ_1 receptor agonists alleviated the $A\beta_{25\text{-}35}$-induced learning impairments in spatial or nonspatial tasks involving short-term as well as long-term memory. These effects were blocked by BD1047, haloperidol, BMY-14,802, and progesterone, all putative σ_1 receptor antagonists (Maurice et al. 1998; Wang et al. 2003; Espallergues et al. 2007; Villard et al. 2009; Yang et al. 2012; Maurice 2016). Of note, whereas they blocked σ_1 receptor agonist effects, the antagonists alone did not alter behavior (positively or negatively) in these models. Agonists of the σ_1 receptor are thus promising agents to treat AD symptoms, with active doses similar to or lower than the reference drugs such as rivastigmine, galantamine, and memantine (Meunier et al. 2006). The symptomatic efficacy of these compounds remains to be confirmed in a transgenic mouse model of AD.

3 Genetic Evidence in Support of a Role for σ_1 Receptors in Neurodegenerative Diseases

The gene encoding the human σ_1 receptor, *SIGMAR1*, is located on chromosome 19 band p13 and contains four exons and three introns. Polymorphisms have been identified that link *SIGMAR1* to vulnerability to or protection against neurological and psychiatric diseases. First, an association with a genetic variant of the σ_1 receptor carrying the mutation E102Q and juvenile amyotrophic lateral sclerosis (ALS) was observed in a consanguineous family, with an autosomal recessive pattern (Al-Saif et al. 2011). This highly conserved mutation among patients is located within a predicted transmembrane domain of the σ_1 receptor. Expression of $SIGMAR1^{E102Q}$ in NSC34 motor neuron-like cells revealed aberrant subcellular distribution of the mutated protein, and cells expressing the mutant protein were more sensitive to apoptosis induced by ER stress (Al-Saif et al. 2011). However, another report suggested that impact of mutations in σ_1 receptor may not be so common in ALS, since only one mutation, T58C, present in the 3′-untranslated region, was identified in a population of 728 Korean ALS patients (Kim et al. 2014). The latter report questioned the causative role of σ_1 receptor mutations in ALS.

Luty et al. (2010) identified, in Australian and Polish patients with frontotemporal lobar degeneration (FTLD) and motor neuron disease (MND), a nonpolymorphic mutation (c.672*51G>T) in the 3′-untranslated region of *SIGMAR1* that increased *SIGMAR1* transcripts in lymphocytes and brain tissue. A morphological examination of the hippocampus showed that overexpression of the σ_1 receptor shunted TDP-43 and fused-in-sarcoma (FUS) proteins from the nucleus to the cytoplasm by 2.3- and 5.2-fold, respectively. Treatment of SK-NMC and SK-N-SH cells with σ_1 receptor ligands significantly altered translocation of TDP-43. The authors concluded that

SIGMAR1 may be a causative gene for familial FTLD-MND with a unique neuropathology that differs from other FTLD and MND cases, and that σ_1 receptor drugs may be potential therapeutic agents for TDP-43/FUS proteinopathies (Luty et al. 2010).

Several studies have suggested links between two polymorphisms identified in populations from different origin and the vulnerability to AD: G241T/C240T and Q2P (Uchida et al. 2005; Huang et al. 2011; Feher et al. 2012). In a population of 239 Japanese patients with AD, these two polymorphisms were in complete linkage disequilibrium with each other resulting in two haplotypes: GC241-240/Q2 and TT241-240/P2. The TT241-240/P2 homozygosity of the *SIGMAR1* gene significantly reduced the risk of AD in apolipoprotein E (apoE) ε4 carriers, suggesting a protective role for this haplotype in AD (Uchida et al. 2005). In a study involving 322 Hungarian late-onset AD patients and 250 elderly control individuals, the polymorphisms also appeared in nearly complete linkage disequilibrium resulting in the two previously observed predominant haplotypes (Feher et al. 2012). An association between the TT241-240/P2 variant and the risk for developing AD was observed. A potential modest interaction of the co-presence of this haplotype with apoE ε4 allele was noted on the risk for AD (Feher et al. 2012). In a study involving an Australian cohort with 82 AD subjects and a Chinese cohort with 330 cases, a significant genetic interaction was found between the apoE ε4 carriers and the P2 haplotype in both populations (Huang et al. 2011). In non-ApoE ε4 carriers, patients with the P2 variant had increased cognitive dysfunction and more neurofibrillary tangles, indicative of an advanced stage of AD. However, in a group of 219 Polish patients with late-onset AD, no significant difference for the *SIGMAR1* allele, genotype, haplotype, and diplotype distributions was observed as compared with the control patients group. Moreover, no interaction with apoE ε4 carriers was found (Maruszak et al. 2007).

Polymorphisms and association analyses have suggested possible interactions in other neuropsychiatric diseases. In a Japanese population, Kishi et al. (2010) described a genetic association between *SIGMAR1* and major depressive disorder (MDD). After selecting the single nucleotide mutation rs1800866 (i.e., the Q2P genotype) in *SIGMAR1* for association analysis, they detected an association of the phenotype (MDD or controls) with the Q2P genotype. However, they found no association between response to serotonin reuptake inhibitor antidepressant treatment and the Q2P genotype. However, the observation suggested that the Q2P genotype may play a role in the pathophysiology of MDD in the Japanese population (Kishi et al. 2010).

Contradictory results have been reported regarding *SIGMAR1* polymorphisms and schizophrenia. Three early studies presented negative results. Ohmori et al. (2000) reported no significant difference in the distribution of the G241T and G240T polymorphisms among 129 schizophrenic patients and 140 controls. Uchida et al. (2003) described no significant association between *SIGMAR1* and schizophrenia in a meta-analysis comprising 636 schizophrenic and 779 control subjects that included previous studies and a case-control association study, between two polymorphisms of *SIGMAR1*, G-241T/C-240T and Q2P, and schizophrenia in a Japanese population. Satoh et al. (2004) analyzed the distribution of *SIGMAR1*

polymorphisms in 100 schizophrenic and 104 control subjects and no significant association was found between the T-485 A, GC-241–240TT, Q2P, and G620A (A211Q) variants and schizophrenia and clinical characteristics. However, more recently, two studies reported evidence in support of an association between the Q2P polymorphism and schizophrenia in Japanese populations. First, Takizawa et al. (2009) analyzed 40 schizophrenic patients and 60 healthy control subjects. In schizophrenics, even after controlling for the effect of medication, the hemodynamic response in the prefrontal cortex of the Q2 genotype group was significantly greater than that of the P2 carriers. Clinical symptoms were, however, not different between the two genetic subgroups. Second, Ohi et al. (2011) did a meta-analysis of the association between the functional Q2P polymorphism and schizophrenia using combined samples, 1,254 schizophrenic patients and 1,574 healthy control subjects from previously published studies, and an additional sampling of 478 patients and 631 controls. They reported evidence in support of an association between Q2P and schizophrenia, without heterogeneity across studies. Patients with schizophrenia showed lower bilateral activation of the prefrontal cortex and P2 carriers had significantly lower activation of the right prefrontal cortex, compared to subjects with the Q2 genotype. Additional evidence, particularly in ethnically diverse populations, is needed. However, these recent studies suggest that certain *SIGMAR1* polymorphisms could be associated with an increased risk of schizophrenia.

Finally, the T485A polymorphism has been implicated in alcoholism. In a population of 307 alcoholic patients and 302 control subjects, Miyatake et al. (2004) observed that the transcriptional activity of the A485 allele and the TT241-240 allele was significantly reduced compared with that of the T485 allele and the GC241-240 allele and that the frequencies of the A485 allele and the TT241-240/P2 haplotype were significantly higher in control subjects compared with alcoholic subjects. They concluded that T485A and GC241-240TT may be functional polymorphisms, and the A485 allele and TT241-240/P2 haplotype are possible protective factors in the development of alcoholism (Miyatake et al. 2004).

4 Pharmacological Evidence That σ_1 Receptor Ligands Engage Neuroprotective Mechanisms in Neurodegenerative Disease

4.1 Alzheimer's Disease

Alzheimer's disease (AD) is the most common form of dementia in the world. According to the World Health Organization, 47.5 million people have dementia, and 7.7 million new cases appear per year. By 2025, the number of people aged 65 and older with AD is estimated to reach 7.1 million in the USA (data from the Alzheimer's Association). AD is clinically characterized by progressive cognitive impairment evolving towards dementia and death. At the physiopathological level, the presence of extracellular senile plaques composed primarily of amyloid-β peptide (Aβ) and the intracellular accumulation of neurofibrillary tangles, due to

aggregation of hyper- and abnormally phosphorylated Tau protein signal the disease and contribute to its complex pathogenesis (Selkoe 2004). Neurodegeneration indeed involves complex synergies between oxidative stress and mitochondrial dysfunction, proteotoxic and cellular stress, calcium imbalance, neuroinflammation, hypoxia, DNA damage, synaptic alterations, and apoptosis. Clinically, the diagnosis is mainly based on cognitive evaluation using the mini-mental status examination (MMSE) score, but magnetic resonance imaging (MRI) of hippocampal volume, visualization of plaques using positron emission tomography (PET), and analyses of blood markers like Aβ species or phosphorylated forms of Tau are being developed as diagnostic tools. With regard to treatment, only symptomatic therapies are currently available for AD. Standard medical treatments include the cholinesterase inhibitors donepezil, rivastigmine, galantamine, or the noncompetitive NMDA receptor antagonist memantine. Psychotropic medications are used to treat secondary symptoms of AD, such as depression, agitation, and sleep disorder. More effective therapeutic agents are needed, and σ_1 receptor ligands may meet some of these needs. However, a better understanding of the σ_1 receptor in the context of AD pathophysiology will be crucial for the discovery and development of effective and potentially curative treatment strategies.

Curative treatment must simultaneously block Aβ species generation (leading ultimately to the formation of senile plaques), prevent the hyperphosphorylation of Tau (responsible for the intracellular accumulation of neurofibrillary tangles), preserve mitochondrial integrity, boost neuritogenesis and dendrite connectivity, and stimulate neurogenesis to repopulate neuronal cells and maintain circuitry. Current medications based on cholinesterase inhibitors or the NMDA receptor antagonist have only demonstrated moderate efficacy in symptom management. A first achievement to enlarge the therapeutic means in AD would be to establish an effective neuroprotective agent. Depending on their impact on Aβ load and Tau hyperphosphorylation, such a compound may help to preserve brain structural integrity and restore altered clearance systems for aggregated amyloid and Tau species. It must, however, present a sufficiently wide mechanism of action to be able to significantly attenuate neurodegenerative disease associated oxidative stress, neuroinflammation, hypoxia, apoptotic pathways, and other processes. Since activation of the σ_1 receptor results in modulation of numerous cytoprotective pathways, σ_1 receptor agonists appeared as promising candidates and, indeed, some of them demonstrated neuroprotective properties in preclinical experimental models of AD. In vitro, the selective σ_1 receptor agonists PRE-084 and (−)-MR22 prevented $A\beta_{25\text{-}35}$-induced toxicity in rat neuronal cultures (Marrazzo et al. 2005). Afobazole, a mixed σ_1 and σ_2 receptor ligand, inhibited the $[Ca^{2+}]_i$ increase in rat cortical neurons after prolonged exposure to $A\beta_{25\text{-}35}$ (Behensky et al. 2013). Afobazole decreased nitric oxide (NO) production in response to $A\beta_{25\text{-}35}$, but did not affect the increase in ROS. The reductions in $[Ca^{2+}]_i$ and NO levels by afobazole were associated with a decrease in neuronal cell death, decreased expression of pro-apoptotic proteins Bax and caspase-3, and increased expression of the anti-apoptotic protein, Bcl-2 (Behensky et al. 2013). Interestingly, microglia also play an important role in σ_1 receptor-mediated cytoprotection against $A\beta_{25\text{-}35}$

toxicity in vitro (Behensky et al. 2013). Treatment with afobazole decreased microglial activation in response to Aβ, as indicated by reduced membrane ruffling and cell migration. It protected against cell death and against the induction of Bax and caspase-3 elicited by prolonged exposure of microglia to $A\beta_{25\text{-}35}$. Afobazole also prevented the decrease in ATP observed in microglia after a 24 h exposure to $A\beta_{25\text{-}35}$. These cytoprotective activities of afobazole were mediated in part by the σ_1 receptor and potentially through purinergic receptors as well (Behensky et al. 2013). These observations support the notion that the cytoprotective effects of targeting the σ_1 receptor not only involve multiple transduction systems and cellular organelles, but also regulate different cellular responses in neuronal and glial cells.

In vivo, the σ_1 receptor agonists PRE-084, (−)-MR22, ANAVEX1-41, ANAVEX2-73, DHEA, DHEA sulfate, and pregnenolone sulfate were neuroprotective in pharmacological models of AD (Meunier et al. 2006; Villard et al. 2009, 2011; Aly et al. 2011; Antonini et al. 2011; Yang et al. 2012; Lahmy et al. 2013). These σ_1 receptor ligands were administered either acutely before or chronically after injection of the pharmacological toxicant ($A\beta_{25\text{-}35}$ peptide or aluminum chloride). The Aβ peptide was injected either intracerebroventricularly or locally into the hippocampal formation, and alone or combined with 192 IgG-saporin, to induce a more severe cholinergic lesion (Antonini et al. 2011). Learning impairment was prevented or attenuated in these models. These beneficial effects were accompanied by neuroprotection. Markers of oxidative stress, cholinergic tonus, neuroinflammation, induction of apoptotic pathways, and cell loss were attenuated by the σ_1 receptor agonists (Meunier et al. 2006; Villard et al. 2009, 2011; Aly et al. 2011; Antonini et al. 2011; Yang et al. 2012). In two studies, the selective σ_1 receptor agonists (−)-MR22 and PRE-084 significantly prevented APP and $A\beta_{1\text{-}42}$ accumulation in the brain following 192 IgG-saporin and/or $A\beta_{25\text{-}35}$ injection (Antonini et al. 2011; Lahmy et al. 2013). Lahmy et al. (2013) also examined activation of the main kinase involved in Tau hyperphosphorylation, glycogen synthase kinase 3β (GSK-3β), and the level of hyperphosphorylated Tau at physiological or pathological epitopes in $A\beta_{25\text{-}35}$-treated mice. PRE-084 and ANAVEX2-73 decreased GSK-3β activation and Tau hyperphosphorylation. Moreover, Fisher et al. (2016) very recently reported that AF710B, a mixed M1 mAChR/σ_1 receptor agonist, administered at 10 μg/kg for 2 months to female 3xTg-AD mice, attenuated learning impairment in the water-maze, decreased BACE1 levels, GSK3β activity, p25/CDK5 levels, and neuroinflammation. AF710B also diminished soluble and insoluble $A\beta_{1\text{-}40}$ and $A\beta_{1\text{-}42}$ accumulation, the number of plaques, and Tau hyperphosphorylation (Fisher et al. 2016). These observations suggested that chronic treatment with a σ_1 receptor agonist can alleviate accumulation of amyloid species and hyperphosphorylated Tau, a prerequisite for an effective neuroprotective and disease-modifying therapeutic agent in AD. Further studies with transgenic animal models will be important to confirm this promising observation with AF710B.

An endogenous ligand for the σ_1 receptor remains unknown. In light of the shifting paradigm that the σ_1 receptor may actually be a chaperone and not a receptor *stricto* sensu, it is conceivable that the assumption that the σ_1 receptor should have a traditionally defined endogenous ligand may be inaccurate.

Nevertheless, several endogenous molecules such as neuropeptides, neurosteroids, and the trace amine *N*,*N*-dimethyltryptamine (Su et al. 1988; Fontanilla et al. 2009) or even physiological changes, like oxidative stress (Meunier and Hayashi 2010) and endoplasmic reticulum (ER) stress (Hayashi and Su 2007), also have been shown to trigger σ_1 receptor activation. This raises the question of whether the σ_1 receptor functions as an endogenous neuroprotection system. To address this question, we combined invalidation of σ_1 receptor expression (using *SIGMAR1* KO mice or repeated NE100 treatment) and induction of amyloid toxicity (using $A\beta_{25\text{-}35}$ injection or cross-breeding with APP_{Swe} mice to generate APP_{Swe}/*SIGMAR1* KO mice) (Maurice et al. 2010, 2015). The intracerebroventricular injection of $A\beta_{25\text{-}35}$ peptide provoked learning deficits and oxidative stress in the hippocampus at lower doses in *SIGMAR1* KO mice compared to wild-type animals (Maurice et al. 2010). When σ_1 receptor expression was absent in APP_{Swe}/*SIGMAR1* KO mice, animals showed significantly decreased survival compared with APP_{Swe} mice, *SIGMAR1* KO mice, and wild-type animals. The spontaneous alternation response of APP_{Swe}/*SIGMAR1* KO animals was lower than single transgenic and control lines between 2 and 12 months of age. Eight-month-old APP_{Swe}/*SIGMAR1* KO mice showed impaired place learning in the water-maze and increased ROS level in the hippocampus, but expression of hippocampal synaptic markers (PSD95, synaptophysin) was unchanged (Maurice et al. 2015). Therefore, it appears that the absence of σ_1 receptor can worsen Aβ toxicity and behavioral deficits.

It must be noted that Yin et al. (2015) reported different results. The authors injected $A\beta_{25\text{-}35}$ in heterozygous *SIGMAR1* KO mice and reported that the peptide injection impaired spatial memory and caused cell death of pyramidal cells in the hippocampal CA1 region of wild-type mice, whereas it did not cause such impairments in heterozygous *SIGMAR1*$^{+/-}$ mice. $A\beta_{25\text{-}35}$ injection in wild-type mice modified the levels of NMDA-activated currents and NR2B phosphorylation in the hippocampal CA1 region in an NE100-sensitive manner. However, the $A\beta_{25\text{-}35}$ injection in *SIGMAR1*$^{+/-}$ mice induced a slight increase in NMDA-activated currents and NR2B phosphorylation. Treatment with PRE-084 caused the same changes in NMDA-activated currents and NR2B phosphorylation as those in $A\beta_{25\text{-}35}$-treated wild-type or *SIGMAR1*$^{+/-}$ mice. These results suggested that partial ablation of σ_1 receptor can reduce $A\beta_{25\text{-}35}$-induced neuronal cell death and cognitive deficits by suppressing $A\beta_{25\text{-}35}$-enhanced NR2B phosphorylation. However, the report by Yin et al. (2015) is not in complete contradiction with Maurice and colleagues (2010, 2015), since Yin et al. did not use homozygous *SIGMAR1* KO mice, which present no σ_1 receptor expression in the forebrain. Rather, these data suggest that the impact of the σ_1 receptor on amyloid toxicity could be complex, depending on levels of σ_1 receptor expression and activity.

Although there is growing evidence in support of the σ_1 receptor as a therapeutic target in AD, the impact of the pathology on the expression level of σ_1 receptor is still poorly documented, particularly in terms of precise densities in the vulnerable brain structures and during the different phases of the disease. Using autoradiography and the non-selective σ_1/σ_2 receptor ligand ^{3}H-DTG, a significant 26% loss of

binding sites was noted in the CA1 *stratum pyramidale* region of the hippocampus of AD patients as compared to healthy controls (Jansen et al. 1993). The loss of σ_1/σ_2 sites correlated with a 29% loss of pyramidal cells. Then, a loss of σ_1 sites was observed using PET imaging in the brain of AD patients (Mishina et al. 2008). The binding potency of ^{11}C-SA4503 was reduced in the frontal lobe, temporal lobe, occipital lobe, cerebellum, and thalamus of early AD patients compared to healthy control subjects, however, this was not observed in the hippocampus (Mishina et al. 2008). It therefore appears that in AD, a decreased level of σ_1 receptor in certain brain regions is associated with specific cell loss in vulnerable cell populations in those regions. However, correlation between the decrease in σ_1 receptor binding potency and pathological stage or its response to a σ_1 receptor agonist-based treatment has not been established.

4.2 Parkinson's Disease

Parkinson's disease (PD) is a progressive multi-system neurodegenerative disease affecting people mainly in later years of life. The prevalence of PD in developed countries is generally estimated at 0.3% of the population and about 1% in people over 60 years of age (de Lau and Breteler 2006). The prevalence increases with age both for men and women (de Rijk et al. 1997). The disease is characterized by specific neuropathological hallmarks. There is formation of abnormal spherical bodies mainly composed of α-synuclein protein, named Lewy bodies, and spindle- or thread-like Lewy neurites in the neuronal soma, starting at precise induction sites and progressing in a topographically predictable sequence within the brain (Braak et al. 2004). Degeneration of dopaminergic nigrostriatal neurons presenting Lewy bodies is regarded as the primary neuropathological correlate of motor impairment in PD, but glutamatergic, cholinergic, GABAergic, tryptaminergic, noradrenergic, and adrenergic neurons may show similar intracellular damage (Braak and Braak 2000). A key pathological factor of PD is mitochondrial dysfunction which is closely related to increased ROS formation. Complex I deficiencies of the respiratory chain account for the majority of unfavorable neural apoptosis generation and are considered one of the primary sources of ROS in PD. It has also been reported that genetic mutations in proteins including α-synuclein, parkin, and phosphatase and tensin homolog induced putative kinase (PINK) are linked to the familial forms of PD. Mutations of these genes have been known to affect mitochondrial function and increase oxidative stress. The exact cause is still undetermined and, although there is presently no cure, treatments such as medication and surgery are used to manage its symptoms (Sveinbjornsdottir 2016). The clinical symptoms in PD are usually defined by motor disturbances but there may be disturbances in several other functions of the nervous system. The symptoms are categorized into motor and non-motor symptoms, and some of them may be provoked or aggravated by the dopaminergic treatment (Sveinbjornsdottir 2016). Evidence that σ_1 receptor activity impacts dopaminergic neurotransmission was initially described in the mid-1980s (Freeman and Bunney 1984; Wachtel and White 1988). More recently,

the σ_1 receptor has been shown to bind dopaminergic psychostimulants such as cocaine and methamphetamine and to be involved in their behavioral and cellular effects including hyperactivity, addiction, and neurotoxicity [for reviews, see (Maurice et al. 2002; Maurice and Romieu 2004; Maurice and Su 2009; Yadid et al. 2010; Robson et al. 2012)]. Analyses of the cellular role of σ_1 receptors in DA neurons confirmed their interest in PD. For instance, Mori et al. (2012) described an association between σ_1 receptors and dopamine (DA)-induced cytotoxicity in Chinese hamster ovary (CHO) cells. Physiologically relevant concentrations of DA provoked apoptosis in *SIGMAR1* knockdown CHO cells and a synergistic conversion of nuclear factor κB (NF-κB) p105 to its active form p50, known to down-regulate the transcription of the anti-apoptotic factor Bcl-2 (Mori et al. 2012). Endogenous σ_1 receptors therefore tonically inhibit the proteasomal conversion/activation of NF-κB induced by physiological DA, suggesting that the σ_1 receptor may be a therapeutic target for the treatment of PD. The mapping of σ_1 receptors in PD was reported by Mishina et al. (2005), using PET imaging with [^{11}C]SA4503. The authors assessed whether σ_1 receptors are altered in the damaged dopaminergic system. The binding potential of [^{11}C]SA4503 appeared significantly lower in the more damaged side of the anterior putamen but with no BP difference between PD patients and controls. DA release was therefore reduced asymmetrically in the putamen of early PD. The authors suggested that [^{11}C]SA4503 PET could be an indicator of presynaptic dopaminergic damage in PD (Mishina et al. 2005). In a recent report, Francardo et al. (2014) treated mice presenting a striatal lesion with 6-hydroxydopamine (6-OHDA), a pertinent pharmacological model of PD, with the σ_1 receptor agonist PRE-084. At 0.3 mg/kg, the drug produced a gradual improvement of spontaneous forelimb use. The behavioral recovery paralleled the increase in DA fiber density in the denervated striatum, a modest recovery of DA levels, and an upregulation of BDNF and GDNF neurotrophic factors and their downstream effectors ERK1/2 and Akt (Francardo et al. 2014). No effect of PRE-084 treatment was observed in *SIGMAR1* KO mice lesioned with 6-OHDA, confirming the pharmacology. Interestingly, σ_1 receptor immunoreactivity was observed in astrocytes and neurons in the substantia nigra and striatum and its intracellular distribution was modified by PRE-084. The σ_1 receptor therefore appeared to regulate an endogenous neuroprotection mechanism and restorative plasticity in experimental PD, suggesting therapeutic potential for effective σ_1 receptor agonists.

However, Hong et al. (2015) reported contradictory results using the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model in mice, a mitochondrial neurotoxicant targeting nigrostriatal DA neurons. The authors proposed that σ_1 receptor *deficiency* could reduce MPTP-induced death of dopaminergic neurons through suppression of NMDA receptor function and DA transporter expression. They used heterozygous and homozygous *SIGMAR1* KO (*SIGMAR1*$^{+/-}$ and *SIGMAR1*$^{-/-}$, respectively) mice and observed that MPTP treatment for 5 weeks in wild-type mice caused motor deficits and DA neurons death in substantia nigra *pars compacta* with an increase in NMDA receptor NR2B phosphorylation. This was not observed in *SIGMAR1* KO mice. The σ_1 receptor antagonist NE100 or the NR2B inhibitor

Ro25-6981 alleviated the motor deficits and death of DA neurons in MPTP-treated wild-type mice. But (MPTP + PRE-084)-treated *SIGMAR1*$^{+/-}$ mice showed similar motor deficits and loss of DA neurons as MPTP-treated wild-type mice. Pharmacological and genetic inactivation of σ_1 receptor suppressed the expression of DA transporter in substantia nigra, and it was corrected by NMDA. PRE-084 enhanced DA transporter expression in wild-type mice or *SIGMAR1*$^{+/-}$ mice (Hong et al. 2015). These data, which contradicted all the previously reported cellular and pharmacological observations, were obtained in a different model as used by Francardo et al. (2014). Since MPTP is a mitochondrial toxicant, a putative direct or indirect interaction of MPTP with σ_1 receptor, localized preferentially at the mitochondria-associated ER membranes (MAM) (Hayashi and Su 2007) and therefore impacting directly mitochondrial physiology, could explain these results. A further characterization of the model is therefore necessary to reconcile the data of Hong et al. (2015) and Francardo et al. (2014).

However, σ_1 receptor agonists appear as the most promising agents for developing neuroprotective treatment strategies, particularly in PD. As it has been reported in animal models, σ_1 receptor ligands are effective in early stages of the disease. It will be important to study what happens in later stages of disease that might shift, inactivate, or otherwise change response of the receptor: is it due to inactivation/involvement of σ_1 receptor in different biochemical process or due to other changes in the cells' requirements of σ_1 receptors associated with progression of the pathology? Furthermore, what is the role of σ_1 receptor in prodromal/preclinical markers of PD, non-motor, extra-nigral symptoms including olfactory and autonomic dysfunctions or cognitive and sleep disturbances?

4.3 Amyotrophic Lateral Sclerosis

Amyotrophic lateral sclerosis (ALS) is the most widespread type of motor neuron disease and has become the third most common neurodegenerative disease in the world (Logroscino et al. 2010). It is a fatal condition clinically presented by progressive weakness, atrophy, and spasticity of muscle tissue reflecting the degeneration of both upper and lower motor neurons in the cortex, brainstem, and spinal cord. There has been no effective therapeutic approach to halt the progression of the disease so far. Currently, symptom management treatments directed at the clinical manifestation of the disease are the only available ALS therapies. Riluzole, a drug that reduces the levels of excitatory neurotransmitter glutamate, is one of the medications which is able to give several months of extended life to patients with ALS (Cheah et al. 2010). Presentation, course, and progression of ALS are heterogeneous. Most cases of the disease are diagnosed based on symptoms, physical signs, electromyography, and tests excluding the overlapping conditions (Hardiman et al. 2011). However, the etiology of ALS is not fully understood and data show that 90% of ALS cases are sporadic cases (sALS). The other 10% are familial cases (fALS), with a Mendelian mode of inheritance. It suggests that genetic factors may play an important role in ALS (Forbes et al. 2004). However in fALS, more than

20 causative genes have been identified and a number of potential causative or disease-modifying genes that have also been described. It remains therefore challenging to detect pathogenic mutations or risk variants for each ALS individual.

Mavlyutov et al. (2013) first demonstrated that the lifespan of the $SOD1^{G93A}$ mouse model of ALS decreases when crossed with *SIGMAR1* KO mice. In the disease, synaptic coverage of motoneuron (MN) somas by C-terminals remained until death while most other synapses retracted (Pullen and Athanasiou 2009). The σ_1 receptor remains in C-terminals of degenerating MN, suggesting that it could act as a halting factor on increased MN excitability, known as one of the pathological hallmarks of ALS (Pambo-Pambo et al. 2009; Mavlyutov et al. 2013). Administration of σ_1 receptor agonists extended the lifespan of $SOD1^{G93A}$ mice (Mancuso et al. 2012). The σ_1 receptor agonist PRE-084 improved locomotor function and motor neuron survival in presymptomatic and early symptomatic mutant $SOD1^{G93A}$ mice (Mancuso et al. 2012). Peviani et al. (2014) tested the efficacy of PRE-084 in a model of spontaneous MN degeneration, the wobbler mouse. Their results demonstrated that PRE-084, caused an increase of BDNF levels in the gray matter, improved motor neuron survival, ameliorated paw abnormality and grip strength performance, modulated astrocytosis and of macrophage/microglia as part of the mechanisms involved in σ_1 receptor-mediated neuroprotection (Peviani et al. 2014). Another σ_1 receptor agonist, SA4503, has been tested in in vitro and in vivo models of ALS. SA4503 prevented $SOD1^{G93A}$-induced cytotoxicity in NSC34 cells and extended the survival time of $SOD1^{G93A}$ mice (Ono et al. 2014). BD1047, a σ_1 receptor antagonist, blocked the SA4503 cytoprotective effect. Expression of $SOD1^{G93A}$ produced mitochondrial dysfunction in NSC34 cells and associated increase in oxidative and ER stress. The σ_1 receptor agonists have demonstrated antioxidant effects in multiple studies and this mechanism may contribute to the cytoprotective effects of σ_1 agonists in MN cell death and neuroprotection in $SOD1^{G93A}$ mutant mice. SA4503 also upregulates the levels of Akt and ERK1/2. For proliferation and maturation of neural precursors the activation of these pathways plays an important role (Li et al. 2001). MN cells in patients with sALS or fALS and SOD1 mutant mice show decreased levels of phosphorylated Akt (Cheah et al. 2010). Fluvoxamine and dehydroepiandrosterone, acting as σ_1 receptor agonists, also induce phosphorylation of Akt. The σ_1 agonists PRE-084 and 4PPBP induce ERK1/2 phosphorylation in neuronal cells (Maurice and Su 2009; Mavlyutov et al. 2011).

The σ_1 receptor is involved in multiple intracellular pathways in neuronal cells. Immunoelectron microscopy studies showed that σ_1 receptors are localized in subsurface cisternae in C-terminals. Muscarinic type 2 acetylcholine receptors (M2 mAChR), voltage-gated potassium channels (Kv2.1), and small conductance calcium-activated potassium channels (SK) channels are located at C-terminals in the postsynaptic plasma membrane. Subsurface cisternae in postsynaptic densities is believed to correlate with postsynaptic hyperpolarization (Fujimoto et al. 1980; Henkart et al. 1976). It also has been shown that in MN, activation of M2 mAChR inhibits SK channels that reduces after hyperpolarization and as a consequence increases excitability (Mavlyutov et al. 2013). *SIGMAR1* KO mice show higher MN

excitability than their wild-type counterparts, consistent with the notion that the absence of σ_1 receptor prevents activation of Kv2.1 and/or SK channels. The mechanism by which the σ_1 receptor regulates and controls excitation in MN likely involves multiple pathways. In C-terminals, the σ_1 receptor is in close physical proximity to Kv2.1 and SK channels, and their interaction has been observed in several cellular responses (He et al. 2012; Mavlyutov et al. 2010). SK channels are activated by an increase in the concentration of intracellular calcium through N-type Ca^{2+} channels.

Calcium dysregulation and excitotoxicity are the predominant mechanisms associated with pathogenesis in ALS (Grosskreutz et al. 2010; Van Den Bosch et al. 2006). It has, for instance, been reported that blood serum from ALS patients induces abnormal NMDA receptor activation (Texido et al. 2011) and that the excitation/inhibition imbalance in MNs of $SOD1^{G93A}$ mice is due to an increased density of glutamatergic synapses, which could lead to enhanced Ca^{2+} influx into cells (Sunico et al. 2011). Control of NMDA receptor hyperactivation may therefore be an effective approach to preventing MN damage. Indeed, σ_1 receptor agonists have been shown to suppress NMDA currents in rat retinal ganglion cells through a PKC-dependent mechanism (Zhang et al. 2011), to prevent Ca^{2+} dysregulation, and to promote neuroprotection in rat cortical neurons by modulating Ca^{2+} influx through NMDA receptors (Lockhart et al. 1995). Furthermore σ_1 receptor ligands can protect MNs in organotypic cultures against excitotoxicity (Guzman-Lenis et al. 2009) and increase PKC-specific phosphorylation of NR1 subunits in spinal MNs (Mancuso et al. 2012). In the latter study, the authors describe that chronic administration of PRE-084 in $SOD1^{G93A}$ mice from 8 to 16 weeks of age can improve the maintenance of the amplitude of muscle action potentials of MNs and locomotor behavior, and preserve neuromuscular connections and MNs in the spinal cord. PRE-084 also extended survival in both female and male mice by more than 15% (Mancuso et al. 2012). The mechanism of action involved an induction of PKC-specific phosphorylation of the NR1 subunit of the NMDA receptor in $SOD1^{G93A}$ animals and a reduction of microglial reactivity (Mancuso et al. 2012). Agonists of the σ_1 receptor may therefore exert a dual therapeutic action by modulating NMDA receptor-dependent Ca^{2+} influx to protect MNs as well as microglial reactivity to ameliorate the MN environment.

4.4 Multiple Sclerosis

Multiple sclerosis (MS) is a progressive demyelinating disease characterized by disseminated lesions within the nervous system, most likely caused by an autoimmune response to self-antigens (Haghikia et al. 2013). Worldwide, there are an estimated 2.5 million patients suffering from MS, with women twice as frequently affected as men. Pathologically, in the early phase of the disease, perivascular inflammatory infiltrates are observed in the brain, optic nerve, and spinal cord. These infiltrates contain mononuclear immune cells, $CD4^+$ and $CD8^+$ T cells, as well as B cells, monocytes, and macrophages. These infiltrates form plaques, the

end stage of inflammation, characterized by demyelination, astrogliosis, and neuronal as well as axonal degeneration (Compston and Coles 2008; Hohlfeld et al. 2016).

The role of dendritic cells, microglia, and macrophages in the immune invasion of the brain is essential. Dendritic cells present antigen to autoreactive T-cells (Lande et al. 2008; Serafini et al. 2006) and microglial cells trigger the inflammatory response. Experimental autoimmune encephalomyelitis (EAE) induced in mice is a clinically relevant animal model that mimicks several aspects of the disease (Gold et al. 2006; Steinman and Zamvil 2005). Recently, Oxombre et al. (2015) used the EAE model to demonstrate the protective effects of a novel σ_1 receptor agonist, chemically based on a tetrahydroisoquinoline-hydantoin structure. EAE was induced in SJL/J female mice by active immunization with myelin proteolipid protein (PLP) [139–151] peptide. A prophylactic treatment with the compound prevented mononuclear cell accumulation and demyelination in brain and spinal cord and increased T2 B-cells and regulatory T-cells, resulting in an overall reduction in the progression of EAE. The authors concluded that the novel σ_1 receptor agonist decreased the magnitude of inflammation in EAE. The effect was associated with increased proportions of B-cell subsets and regulatory T-cells (Oxombre et al. 2015).

4.5 Huntington's Disease

Huntington's disease (HD) is an autosomal, dominantly inherited neurodegenerative disease, caused by an expansion of cytosine–adenine–guanine (CAG) repeats in the first exon of the huntingtin gene, which encodes the huntingtin (Htt) protein (Dorsey et al. 2013). The inherited mutation results in production of an elongated polyQ mutant huntingtin protein (mHtt). The expansion of CAG repeats leads to the formation of intracellular and intranuclear aggregates in affected neurons (Orr et al. 1993). Patients with HD rapidly develop severe mental and physical disability due to brain atrophy and loss of neurons in the striatum and cerebral cortex (Huntington 1872). No cure or treatment to prevent the progression of HD is currently available. The cellular Htt protein is expressed in most tissues and is involved in protein trafficking, postsynaptic signaling, vesicle transport, transcriptional regulation, and regulation of cell death. Accumulation of the mHtt variant results in alteration of gene transcription, energy production, dysregulation of neurotransmitter metabolism, and activation of intracellular pathways, particularly those leading to ER stress (Reijonen et al. 2008). ER stress and oxidative damage are linked through close communication between the ER and mitochondria. Both play a major role in the neurodegenerative processes in HD (Gil and Rego 2008; Reijonen et al. 2010).

Several recent studies have shown that activation of σ_1 receptor may play a neuroprotective role in HD. First, Hyrskyluoto et al. (2013) showed in an in vitro study using PC6.3 cells overexpressing mHtt that PRE-084 counteracted the toxicity and increased the antioxidative and anti-apoptotic responses of the cells. The cytoprotective effect of PRE-084 involved an upregulation of calpastatin and

induction of the NF-κB pathway (Hyrskyluoto et al. 2013). Second, Miki et al. (2015) showed that accumulation of σ_1 receptor is observed in the nuclear inclusions seen in HD. Using HeLa cells transfected with N-terminal mHtt, they observed that cells harboring mHtt produced σ_1 receptor-positive nuclear inclusions. Small interfering-RNA targeting σ_1 receptor (*SIGMAR1* siRNA) and epoxomicin a specific inhibitor of the proteasome, significantly impaired accumulation of aggregates in the cytoplasm and nucleus. Leptomycin B, a specific inhibitor of exportin 1, also provoked nuclear inclusions. Htt became insoluble after treatments with *SIGMAR1* siRNA and epoxomicin. Proteasome activity increased concurrently along with Htt accumulation but was reduced in *SIGMAR1* siRNA-transfected cells. In contrast, overexpression of σ_1 receptor was associated with decreased number and size of mHtt-containing nuclear inclusions (Miki et al. 2015). However, in this study, the σ_1 receptor agonist PRE-084 and antagonist BD1063 had no effect on cellular viability and proteasome activity. Nevertheless, these findings suggested that in HD the ubiquitin–proteasome pathway is implicated in nuclear inclusion formation, and that the σ_1 receptor participates in the degradation of aberrant proteins in the nucleus via ER-associated degradation machinery.

Third, pridopidine's effect in HD has recently been proposed to involve mainly its agonist action at σ_1 receptors. Pridopidine (4-[3-methanesulfonyl-phenyl]-1-propyl-piperidine), formerly known as ACR16, is a compound from the phenyl-piperidine group of molecules, known as "dopamine stabilizers" or "dopidines" (Pettersson et al. 2010). This class of compounds is being widely investigated in neurodegenerative diseases, including HD (Feigin 2011; Reilmann 2013). Pridopidine is structurally related to 3-(3-hydroxyphenyl)-N-n-propylpiperidine (3-PPP), a racemate whose enantiomers have different effects on dopamine receptors. The (+)3-PPP enantiomer is a weak agonist while the (−)3-PPP enantiomer is a weak antagonist (Mulder et al. 1985). Both drugs have high affinity for the σ_1 receptor, with (+)3-PPP having greater σ_1 receptor binding affinity than (−)3-PPP (Largent et al. 1986a, b; McCann and Su 1991). The structurally related compound (S)-(−)-3-(3-methanesulfonyl-phenyl)-1-propyl-piperidine (OSU6162) binds σ_1 receptors with nanomolar affinity (Sahlholm et al. 2013) and occupies σ_1 receptors rather than dopamine D2 receptors at behaviorally active doses. The authors report a 57% reduction in [^{11}C]SA4503 binding in rats with a dose of 3 mg/kg OSU6162 and 85% inhibition of [^{11}C]SA4503 binding with 15 mg/kg pridopidine, compared to only a 44–66% reduction of [^{11}C]raclopride binding to D2 receptors was observed at 60 mg/kg OSU6162 (Sahlholm et al. 2015). The neuroprotective effect of pridopidine in HD was evaluated using in vivo and in vitro models by Squitieri et al. (2015). These models comprise R6/2 transgenic mice expressing exon 1 of human Htt with approximately 160 CAG repeats and conditionally immortalized mouse striatal knock-in cells expressing endogenous levels of wild-type (STHdh7/7) or mHtt (STHdh111/111). In these models, pridopidine protected cells from apoptosis and improved motor performance and prolonged lifespan in R6/2 mice. The drug enhanced expression of BDNF and DARPP32 in the striatum of the transgenic mice and induced remodelling of mHtt

aggregates. The anti-apoptotic effect was due to enhancement of ERK activation and was blocked by NE100, indicating that the neuroprotective actions of pridopidine are σ_1 receptor-mediated. NE100 alone had no effect on cell survival or signaling in this study. Taken together, these findings support the idea that compounds with affinity for the σ_1 receptor have neuroprotective and disease-modifying properties in HD models and represent potential therapeutic agents for effectively treating the disease.

5 Conclusions

Accumulating evidence supports the notion that selective and non-selective σ_1 receptor ligands may be effective therapeutic agents for neurodegenerative diseases. Inactivation of the σ_1 receptor in animal models exacerbated the pathology and genetic studies suggested that some polymorphisms in the *SIGMAR1* gene could potentiate or appear as risk factors affecting the vulnerability to develop the pathology. These findings are concordant with the notion that σ_1 receptor activity is an important determinant of neuroprotection. Described as a potential *endogenous neuroprotection system* or as a *pluripotent modulator in living system* by some authors (Maurice and Su 2009; Su et al. 2016), the actions of the σ_1 receptor may in fact rely on its association with multiple cellular pathways or at least a complex mode of action. Direct modulation of neurotransmitter activity or second messenger systems, regulation of trophic factors and cytokine activities, modulation of local Ca^{2+} mobilization, preservation of mitochondrial integrity, regulation of transcription factors and gene expression, and activation of ER stress pathways are among the cellular processes affected by σ_1 receptor modulation. The present review has highlighted that PRE-084 is among the most extensively published σ_1 receptor ligands in neurodegenerative disease so far and may serve as a reference compound for future drug discovery and development. It has been reported to be efficacious at 0.3–1 mg/kg doses in models of amnesia, AD, PD, ALS, and HD. Its in vitro and in vivo effects have been described in more than a hundred publications, many of which report comparator data against drugs that are currently in clinical trials, including igmesine, SA4503, and ANAVEX2-73. Finally, a phase II clinical trial is in progress with a σ_1 receptor drug in AD. Other trials may be initiated in the near future and the data generated will be crucial to validate the σ_1 receptor as a drug target in neurodegenerative diseases and better understand the real clinical potential of σ_1 receptor ligands as therapeutic agents in cognitive impairments and neurodegenerative pathologies.

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Sigma-1 Receptor and Neuronal Excitability

Saïd Kourrich

Contents

Abstract

The sigma-1 receptor (Sig-1R), via interaction with various proteins, including voltage-gated and ligand-gated ion channels (VGICs and LGICs), is involved in a plethora of neuronal functions. This capability to regulate a variety of ion channel targets endows the Sig-1R with a powerful capability to fine tune neuronal excitability, and thereby the transmission of information within brain circuits. This versatility may also explain why the Sig-1R is associated to numerous diseases at both peripheral and central levels. To date, how the Sig-1R chooses its targets and how the combinations of target modulations

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alter overall neuronal excitability is one of the challenges in the field of Sig-1R-dependent regulation of neuronal activity. Here, we will describe and discuss the latest findings on Sig-1R-dependent modulation of VGICs and LGICs, and provide hypotheses that may explain the diverse excitability outcomes that have been reported so far.

Keywords
AMPA receptor • Auxiliary subunit • Calcium channels • Chaperone protein • Intrinsic excitability • NMDA receptor • Potassium channels • Sigma-1 receptor • Sodium channels • Voltage-gated ion channels

1 Introduction

Brain function is governed by an extraordinarily complex, yet organized, neuronal network wherein information transmission is controlled by constant interactions between synaptic and intrinsic cellular excitability factors (Fig. 1). The first, synaptic excitability, controls the transmission of chemical signals at synapses. These chemical signals are heavily influenced by excitatory (glutamate) and inhibitory (γ-Aminobutyric acid, GABA) neurotransmitters that will bind to postsynaptic glutamate (i.e., mainly AMPA and NMDA receptors, AMPARs and NMDARs, respectively) and GABA receptors (GABAR), respectively. The second, intrinsic excitability, translates these chemical signals into an electrical signal that will travel, if strong enough to generate action potentials, to axon terminals and induce the release of neurotransmitters (i.e., chemical signals) and thereby convey information to the next neuron. Altogether, these two factors work in concert to generate global neuronal excitability. Conceptually, intrinsic excitability factors include any elements located on the soma, dendrites, or axon that are "remote" from the synapse but either passively or actively modulate membrane excitability. For the sake of brevity, this chapter will refer only to the elements contributing to the active membrane properties, i.e., the generation of action potentials and the characteristics of repetitive firing processes that are mainly controlled by the interplay between voltage-gated sodium (Na^+), potassium (K^+), and calcium (Ca^{2+}) channels (Hille 2001). Because ion channels occupy various but nonetheless specific subcellular compartments (Lujan 2010; Bredt and Nicoll 2003), their subcellular localization and functional states alter various aspects of neuronal transmission, including action potential generation, conduction along the axon, neurotransmitter release, and postsynaptic receptor sensitivity. During each of these steps, various types of ion channels open and close in a timely and coordinated manner, a process that shapes action potentials waveform, frequency, and thereby appropriately convey information. To ensure appropriate information transmission, neurons rigorously regulate the function, transcription, translation, and subcellular targeting of these ion channels to the right final destinations. Ion channel auxiliary subunits play a key role in these functions, e.g., Kvβs and KChips (Kv channel-interacting proteins) for K^+ channels (Vacher et al. 2008; Maffie and Rudy 2008); $Ca_v\alpha_2\delta$, $Ca_v\beta$, and $Ca_v\gamma$

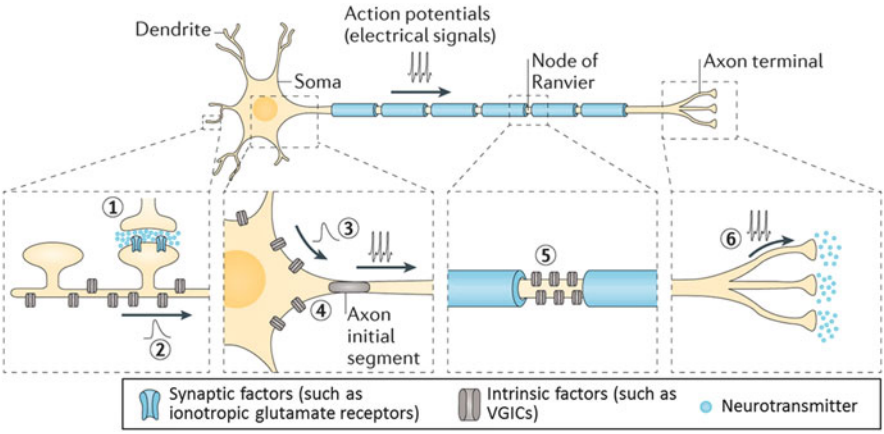

Fig. 1 Compartmentalization of synaptic and intrinsic excitability factors within the cell. A signal received by a neuron through presynaptic neurotransmitter release (1) travels through successive subcellular compartments before it can transmit the information to the next neuron. At the synaptic level (1), glutamate, via activation of AMPARs and NMDARs (synaptic excitability factors), generates an excitatory postsynaptic potential (EPSP) that is influenced by intrinsic factors (such as VGICs: K^+, Na^+, and Ca^{2+} channels) as it travels along the dendrite (2), soma (3), axon hillock and the axon initial segment (4) (compartment rich in Na^+ channels). If an EPSP is strong enough to depolarize the membrane to action potential threshold, then action potentials are generated and will be further influenced by intrinsic factors, for example those located at the nodes of Ranvier (5), as they travel along the axon, until they reach the axon terminal (6), where they will trigger neurotransmitter release. Modulation of ion channel function at any of these steps can result in plasticity of intrinsic excitability, and thereby alter the generation or conduction of action potentials. (Reproduced from Kourrich et al. 2015)

subunits for Ca^{2+} channels (Arikkath and Campbell 2003; Vacher et al. 2008); $Na_v\beta$ for Na^+ channels (Calhoun and Isom 2014; Vacher et al. 2008); and stargazin for AMPARs (Bredt and Nicoll 2003). While these auxiliary subunits have been extensively studied, the regulatory power of the Sig-1R, emerging as a new auxiliary subunit (Aydar et al. 2002), is scarcely understood. To date these regulatory functions have been investigated mostly using heterologous expression systems, including cell culture models and Xenopus oocytes (Aydar et al. 2002; Crottes et al. 2011; Kinoshita et al. 2012; Kourrich et al. 2012). Because little, if any, is known about the regulatory functions of the sigma-2 receptor (Sig-2R) subtype, this chapter discusses findings from Sig-1R's studies and will use the term Sig-R when the receptor subtype was not identified. Furthermore, although the Sig-1R can also modulate a plethora of other neurotransmitter and neuromodulator systems, including dopaminergic (Fishback et al. 2010; Moreno et al. 2014; Navarro et al. 2010, 2013), serotoninergic (Bermack and Debonnel 2001; Fishback et al. 2010), histaminergic (Moreno et al. 2014), and cholinergic (van Waarde et al. 2011), for brevity, this chapter focuses on direct interactions of Sig-1Rs with

VGICs and LGICs that are directly relevant to neuronal excitability and synaptic transmission.

The first section, supported by recent data, raises the hypothesis that the Sig-1R is an atypical auxiliary regulatory subunit for ion channels, and particularly for VGICs. The second and third sections summarize the state of knowledge on Sig-1R-dependent regulation of LGICs and VGICs, respectively. And the fourth section discusses mechanisms that could explain the observed effects of Sig-1Rs activity on neuronal intrinsic and synaptic excitability, and thus, how they affect overall neuronal activity.

2 The Sig-1R: An Atypical Auxiliary Subunit for Ion Channels

Auxiliary subunits are non-conducting, modulatory components of the multi-protein ion channel complexes that underlie normal neuronal signaling. Although the concept of auxiliary subunits has not been clearly defined, they usually fulfill several criteria, such as the capability to: (1) directly modulate the biophysical properties of the α pore-forming subunits; (2) participate to the assembly, trafficking and surface expression of the pore-forming subunits; (3) regulate ion currents in ligand-independent manner; and (4) alter pharmacological interactions or bind drugs directly. The Sig-1R fulfills all of these criteria. It directly modulates the biophysical properties of channels (Aydar et al. 2002; Kinoshita et al. 2012; Zhang et al. 2009), contributes to trafficking and surface distribution of channels (Balasuriya et al. 2014; Crottes et al. 2011; Kinoshita et al. 2012; Kourrich et al. 2013), regulates ion currents in ligand-dependent and independent manner (Aydar et al. 2002; Kinoshita et al. 2012), binds drugs directly (Hayashi et al. 2011; Kourrich et al. 2012), and directly interacts with the pore-forming subunit (Balasuriya et al. 2012, 2013, 2014). However, indirect evidence suggests that other typical criteria that define auxiliary subunits, including its presence in purified channel complexes and both stable and integral association with the pore-forming α subunits, are not shared by the Sig-1R. For example, in both heterologous expression system (NG108-15 cell line) and in brain tissue, only a marginal level of Sig-1Rs coimmunoprecipitated with native Kv1.2 α pore-forming subunits (Kourrich et al. 2013).

Although these functional characteristics support the idea that the Sig-1R is an auxiliary subunit for voltage-gated ion channels (VGICs), other characteristics seem to belong exclusively to the Sig-1R, which makes this chaperone protein atypical and unique in its kind. Typically, VGIC auxiliary subunits are transmembrane or cytoplasmic proteins located at the plasma membrane or in its vicinity (i.e., plasmalemma level) (Vacher et al. 2008). In contrast, the Sig-1R is present in various subcellular compartments, including plasmalemma, plasma membrane, nucleus, mitochondrial and ER membrane, and extracellular space (Hayashi and Su 2003, 2007; Su et al. 2010; Luty et al. 2010; Shioda et al. 2012). Furthermore, while ion channels auxiliary subunits are commonly associated to specific ion channel subfamilies, the Sig-R associates with channel proteins from very different

classes and superfamilies (VGICs and LGICs) (Kourrich et al. 2012). However, recent studies showed that in some cases, other ion channel auxiliary subunits exhibit these unique features as well. For example, NCA localization factor-1 (NLF-1) functions as a unique Na^+ leak channel auxiliary subunit that is located at the ER level (Xie et al. 2013), and emerging evidence suggests, although rarely reported, that the regulatory functions of channel family-specific auxiliary subunits can be extended to other channel superfamilies. For example, the Na^+ channel auxiliary subunits $Na_v\beta$ modulate voltage-gated K^+ channels (Calhoun and Isom 2014), e.g., $Na_v\beta1$ regulates cell-surface expression of the voltage-gated K^+ channel Kv4.2 (Marionneau et al. 2012).

To date, Sig-1R has been shown to associate and directly regulate both VGICs that belong to all superfamilies (Na^+, K^+, and Ca^{2+}) and ionotropic glutamate receptors (NMDARs), which make the Sig-1R a powerful and pluripotent regulator of neuronal activity, from synaptic transmission to intrinsic excitability.

3 Synaptic Transmission (Ligand Gated Ion Channels, LGICs): Pre- and Postsynaptic Evidence

Mechanistically, excitatory synaptic transmission depends on both presynaptic glutamate release and postsynaptic ionotropic glutamate receptors functions. Although it is evident that Sig-1Rs modulate excitatory transmission in both central (CNS) (Bergeron et al. 1993; Bermack and Debonnel 2005; Monnet et al. 1990; Yamamoto et al. 1995; Liang and Wang 1998; Zhang et al. 2011) and peripheral nervous systems (PNS) (Kim et al. 2008; Yoon et al. 2010), the cellular mechanism and the site of action through which and where the Sig-1R operates are less clear. This could reflect, as discussed below, subcellular-specific actions of Sig-1Rs on pre- or postsynaptic receptors – actions that would depend on the brain region and on the mechanisms of action, i.e., direct versus indirect (i.e., via second messenger systems).

To date and historically, most of the studies revealed a role for the Sig-1R in the regulation of NMDAR-mediated transmission and some evidence supports a role for the Sig-1R in the regulation of $GABA_A$R- (GABA type A receptor) (Zheng 2009; Mtchedlishvili and Kapur 2003) and AMPAR-mediated transmission (Liang and Wang 1998; Meyer et al. 2002; Ohi et al. 2011). Briefly, although selective antagonists were not yet available, pharmacological studies using combination of non-selective Sig-Rs ligands suggest that the Sig-1R has the potential to modulate NMDAR-mediated transmission bidirectionally. These modulations occur in both the CNS and PNS, including the CA3 field of rat dorsal hippocampus (Bergeron et al. 1993; Bermack and Debonnel 2005; Monnet et al. 1990), cultured neuronal cells from fetal rat telencephalon (Yamamoto et al. 1995), pyramidal cells of medial prefrontal cortex (Liang and Wang 1998), spinal cord (Kim et al. 2008; Yoon et al. 2010), and retinal ganglion cells (RGCs) (Zhang et al. 2011).

3.1 Presynaptic Mechanisms

Via a Sig-1R-dependent mechanism, endogenous neuroactive steroid pregnenolone sulfate (PREGS) enhances the frequency but not the amplitude of AMPAR-mediated miniature excitatory postsynaptic currents (mEPSCs) in cultured hippocampal neurons (Meyer et al. 2002). This effect was accompanied by a decrease in paired-pulse facilitation, an electrophysiological phenotype that indicates enhanced probability of presynaptic glutamate release. This adaptation was prevented by pertussis toxin-induced blockade of Gi/o-coupled receptor and dependent on elevation of intracellular Ca^{2+}. Interestingly, this effect is reminiscent of Dong et al. (2007) study, which showed that Sig-1R activation by dehydroepiandrosterone sulfate (DHEAS), another endogenous neuroactive steroid, promotes presynaptic glutamate release in the rat prelimbic cortex via activation of dopamine 1 receptor (D1R) (Dong et al. 2007). At a first glance, because D1R is coupled to Gs and not to Gi/o, these two adaptations appear to be mediated by different mechanisms. However, one way to reconcile these seemingly contradictory findings is to examine the particular G protein coupling with metabotropic heteromeric complexes. For example, Sig-1Rs via heteromeric complexes modulate several presynaptic metabotropic receptors that play a role in presynaptic glutamate release, e.g., dopamine 1 receptor (D1R) (Ferraro et al. 2012), 5-HT receptors (Marek and Aghajanian 1998; Hawkins 2013), and histamine H3 receptor (H3R) (Brown et al. 2001; Brown and Reymann 1996). The latter, H3R, is coupled to Gi/o and depending on brain regions can be located predominantly on pre- or postsynaptic side (Brown et al. 2001; Ellenbroek 2013). H3R and D1R can heterodimerize, and these complexes are coupled with Gi (Ellenbroek 2013). Importantly, through direct binding, Sig-1R activation regulates D1R-H3R signaling (Moreno et al. 2014). Although the signal transduction triggered by activation of the Sig-1-D1-H3 receptor complex is still unclear, this complex represents an interesting molecular candidate for Sig-1R-dependent modulation of presynaptic glutamate release.

3.2 Postsynaptic Mechanisms

(+)-SKF 10,047 activation of Sig-1Rs suppresses NMDAR- but not AMPAR-mediated evoked EPSCs in different types of RGCs. Because this mechanism was not accompanied by changes in AMPAR-mediated miniature EPSCs, it was interpreted that Sig-1Rs activation did not alter spontaneous presynaptic release of glutamate (Zhang et al. 2011). Consistent with postsynaptic mechanism, combining pharmacological and electrophysiological approaches in brain slices, Zhang et al. (2012) reported that methylphenidate (i.e., Ritalin), a drug used for Attention Deficit Hyperactivity Disorder (ADHD), enhances NMDAR- but not non-NMDAR (presumably AMPAR)-mediated transmission (Zhang et al. 2012). This effect required PLC/IP3/PKC signaling pathway and was independent of catecholamine release or methylphenidate-induced increase in the concentration of DA and norepinephrine in the synaptic cleft, and therefore, likely involves a post-synaptic

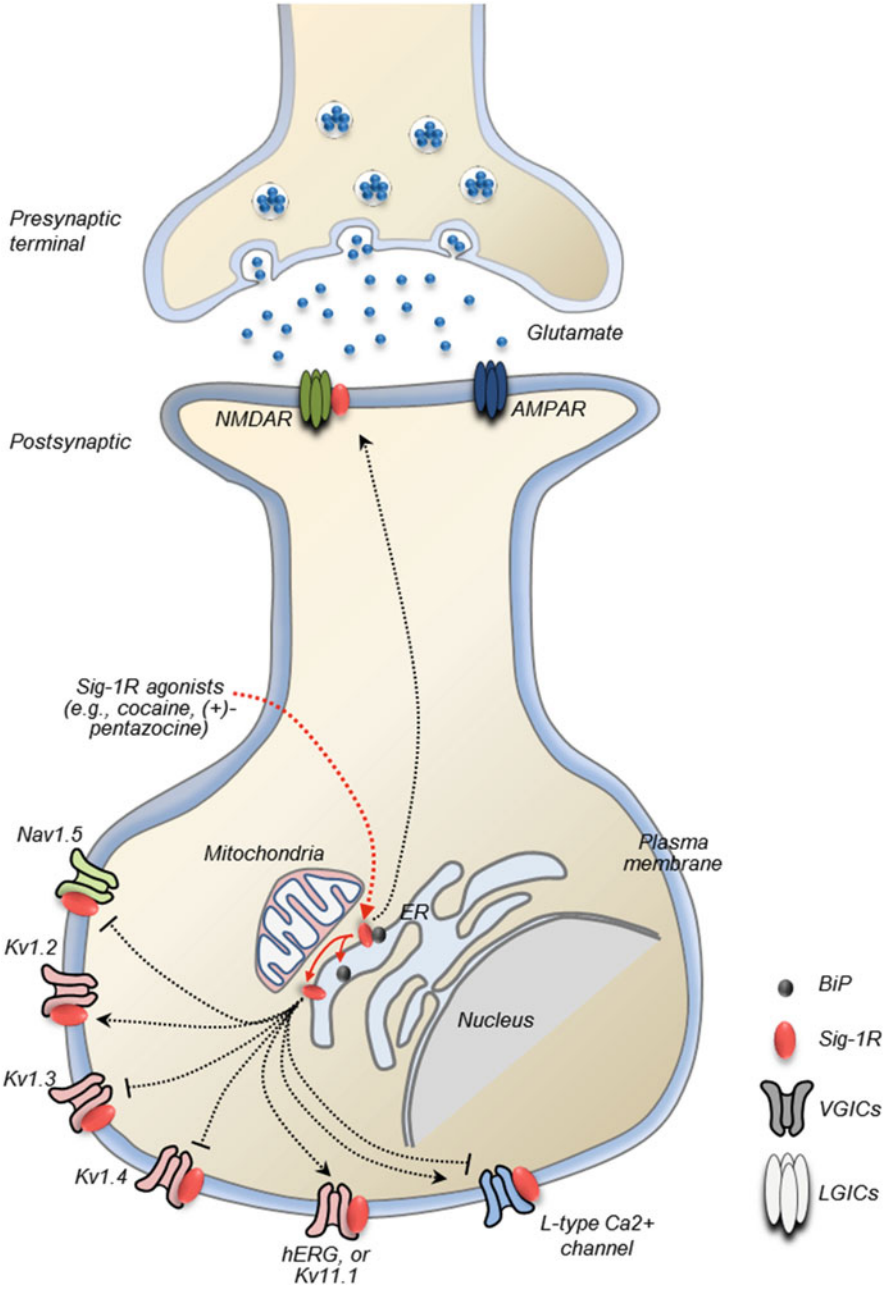

Fig. 2 Schematic illustrating direct regulation of Na^+, Ca^{2+}, and K^+ voltage-gated ion channels by the sigma-1 receptor (Sig-1R). Upon ligand stimulation (e.g., cocaine, (+)-pentazocine, PRE-084), Sig-1Rs dissociate from binding immunoglobulin protein (BiP) (i), another endoplasmic reticulum (ER) chaperone protein, and then translocate from the mitochondrion-associated ER membrane

Fig. 2 (continued) (MAM, interface between mitochondrion and ER) to the ER and plasmalemma. Acting as an interorganelle signaling modulator, Sig-1R regulates a variety of functional proteins, both directly and indirectly. Here are represented only the regulations mediated by direct interaction with Sig-1R protein targets. Dashed pointed and flathead arrows indicate positive and negative regulations, respectively. On the one hand, Sig-1R can upregulate ion channel expression at the plasma membrane either through the regulation of subunit trafficking activity (hERG) (Crottes et al. 2011) or a mechanism that is still unidentified (Kv1.2) (Kourrich et al. 2013). Sig-1R activation by (+)-SKF10,047 can also enhance binding with NMDARs, a mechanism that may play a role in NMDAR subunit trafficking to the cell surface (Balasuriya et al. 2013; Pabba et al. 2014). On the other hand, Sig-1R can inhibit ion currents through modulation of target biophysical properties (Kv1.3, Kv1.4) (Aydar et al. 2002; Kinoshita et al. 2012) and likely trafficking mechanisms ($Na_v1.5$) (Johannessen et al. 2009) (Balasuriya et al. 2012). This can occur through both ligand-independent (Kv1.3, Kv1.4) (Aydar et al. 2002; Kinoshita et al. 2012) and ligand-dependent mechanisms (Kv1.4) (Aydar et al. 2002). Interestingly, Sig-1R can both enhance (Sabeti et al. 2007) and inhibit (Tchedre et al. 2008) L-type Ca^{2+} current; however, it is still unknown whether these two opposing effects are both mediated through a protein–protein interaction mechanism

mechanism. It is only recently that a study using atomic force microscopy (AFM) imaging demonstrated that Sig-1Rs can interact directly with NMDARs (Balasuriya et al. 2013) (Fig. 2). However, the nature and subunit-specificity of this interaction remain unclear. While this study found that the Sig-1R interacts with NMDARs via binding with GluN1 subunit (and not GluN2A) (Balasuriya et al. 2013), another study, using a less stringent approach, found that the Sig-1R coimmunoprecipitates with GluN2 subunits in rat hippocampus (Pabba et al. 2014). Because GluN1 antibody was not used, Sig-1R indirectly binding with GluN2 through direct binding with GluN1 cannot be excluded. Interestingly, they have also found that Sig-1R activation by (+)-SKF10,047 enhances this interaction, a mechanism that appears to play a role in NMDAR subunits trafficking to the cell surface.

In summary, the Sig-1R can modulate excitatory glutamate transmission via both pre- and postsynaptic mechanisms. Depending on the subcellular site of action, Sig-1R activation can either lead to changes in mechanism that regulate presynaptic glutamate release or modulate NMDA receptor activity via direct protein–protein association on the postsynaptic side. However, it is still unknown whether the functional relationship between the Sig-1R and NMDARs is constrained to the regulation of subunits trafficking to the surface or involves the modulation of NMDAR function as well.

4 Neuronal Intrinsic Excitability: Focus on VGICs

Sig-1R-dependent modulations of excitatory glutamate transmission will directly alter the capability of the neuron to generate an excitatory postsynaptic potential (EPSP). This EPSP is influenced by intrinsic factors (such as VGICs: K^+, Na^+, and Ca^{2+} channels) as it travels along the dendrite, soma, axon hillock, and the axon initial segment (compartment rich in Na^+ channels). At this level, if an EPSP is

Table 1 Summary of direct effects of Sig-1R activation on VGICs

Functional effects	Experimental system	Evidence	Refs
Ca^{2+} currents			
↓N, L, P/Q and R-type	Parasympathetic intracardiac neurons; superior cervical ganglia (cell culture)	2nd messenger systems and G proteins not required	Zhang and Cuevas (2002)[a]
↓L-type	Retinal ganglion cells (cell culture)	co-IP	(Tchedre et al. 2008)
Na^+ currents			
↓$Na_v1.5$	Cardiac myocytes; cell lines (cell culture)	2nd messenger systems and G proteins not required; AFM	(Johannessen et al. 2009; Fontanilla et al. 2009; Balasuriya et al. 2012)
K^+ currents			
↓I_A	Neurohypophysial terminals (pituitary gland slices)	2nd messenger systems and G proteins not required	(Lupardus et al. 2000)
↓$I_{K(DR)}$, I_{BK}	Parasympathetic intracardiac neurons (cell culture)	2nd messenger systems and G proteins not required	(Zhang and Cuevas 2005)
↓I_A (Kv1.4)	Xenopus oocytes; rat posterior pituitary gland	co-IP	(Aydar et al. 2002)
↑I_{hERG}	Xenopus oocytes; HEK cells; human K562 myeloid leukemia cells	co-IP; AFM	(Crottes et al. 2011; Balasuriya et al. 2014)
↓Kv1.3	Xenopus oocytes; HEK 293 cells	co-IP	(Kinoshita et al. 2012)
↑Kv1.2	Brain tissue (NAc and PFC); cell lines (NG108-15 and Neuro2A)	co-IP	(Kourrich et al. 2013)

AFM atomic force microscopy, *co-IP* coimmunoprecipitation, I_A A-type K+ current, I_{BK} large-conductance Ca2+-activated K+ current, I_{hERG} human ether-à-gogo K+ current, $I_{K(DR)}$ delayed outwardly rectifying K+ current

[a]Rank order potency of various Sig-R ligands suggests these effects may be through Sig-2R

strong enough to depolarize the membrane to action potential threshold, then action potentials are generated and will be further influenced by intrinsic factors as they travel along the axon until they reach the axon terminal where they will trigger neurotransmitter release. Through both indirect and direct physical interaction the Sig-1R regulates various VGICs, and thereby has the capability to modulate both the generation and the conduction of action potentials. This section summarizes evidence supporting direct regulation of VGICs by the Sig-1R and its resulting effects on ion channel functions (Table 1, Fig. 2).

4.1 Voltage-Gated Ca^{2+} Channels

Ca^{2+} controls neuronal activity both directly and indirectly. For example, influx of Ca^{2+} from extracellular to intracellular compartments through voltage-gated Ca^{2+} channels triggers activity-dependent neurotransmitter release at the synaptic level. Ca^{2+} can also act as a second messenger to trigger specific intracellular signaling pathways. Overall, while Na^{+} and K^{+} channels are involved in processes requiring fast transduction signal, Ca^{2+} plays a role in both fast synaptic transmission and slow changes in neuronal function through the regulation of intracellular signaling pathways (Catterall 2010; Dolphin 2009). Sig-Rs strongly modulate intracellular Ca^{2+} concentration in both neuronal and non-neuronal cells, a process that can occur through the regulation of both Ca^{2+} entry through the plasma membrane and Ca^{2+} mobilization from endoplasmic stores (Hayashi et al. 2000; Hayashi and Su 2001) [for reviews see (Fishback et al. 2010; Maurice and Su 2009; Su et al. 2010)]. This regulatory function is mediated through either direct or indirect action of the Sig-1R on Ca^{2+} channels. Probably the first studies that have suggested that Sig-Rs (Sig-1R and Sig-2R were not distinguished yet) can be associated to Ca^{2+} channels, and thereby directly modulate their functions, came from indirect evidence in the 1990s (Brent et al. 1997; Church and Fletcher 1995; Rothman et al. 1991). Using binding, pharmacological and electrophysiological assays, the effects of various Sig-R ligands were tested on neuronal Ca^{2+} dynamics. However, it was unclear whether these inhibitory actions on Ca^{2+} dynamics were mediated by the Sig-1R, Sig-2R, or both, or whether Sig-R ligands used at high concentrations (i.e., micromolar) were acting directly on Ca^{2+} channels rather than on Sig-Rs. It was until the discovery of highly selective toxins for specific voltage-gated Ca^{2+} channels that it became evident that Sig-Rs could be both associated with and regulate voltage-gated Ca^{2+} channels (Brent et al. 1997).

To date, the L-type voltage-gated Ca^{2+} channel is the only one to have been identified as a direct target for the Sig-1R in the nervous system (Tchedre et al. 2008). On the one hand, data from cultured RGCs provided unequivocal evidence, via co-immunoprecipitation assays, for physical interaction between Sig-1Rs and L-type Ca^{2+} channels (Fig. 2, Table 1). In this preparation, the Sig-1R agonist (+)-SKF10,047 directly inhibited Ca^{2+} currents – an effect prevented by the Sig-1R antagonist BD1047 (Tchedre et al. 2008). On the other hand, using brain slices preparation, PREGS activation of Sig-1Rs triggered an L-type Ca^{2+} channel-dependent LTP in CA1 region of the hippocampus. This form of plasticity was NMDAR-independent but dependent on Sig-1R-induced increase in L-type Ca^{2+} currents (Sabeti et al. 2007). The Sig-1R antagonist BD1047 prevented PREGS facilitation of L-type Ca^{2+} channel-dependent LTP and mimicked the effect of nimodipine, a specific L-type Ca^{2+} channel blocker (Sabeti et al. 2007).

Although this section focuses on Ca^{2+} currents mediated by voltage-gated Ca^{2+} channels directly involved in the regulation of neuronal excitability, it is worth noting that Sig-1R also binds and regulates non-voltage-gated Ca^{2+}-permeable channels. This includes: (1) regulation of IP3 receptors [reviewed in (Fishback et al. 2010; Maurice and Su 2009; Su et al. 2010)], which result in proper ER to

mitochondria Ca^{2+} signaling and thought to regulate mitochondrial bioenergetics (Hayashi and Su 2007) [reviewed in (Fishback et al. 2010; Su et al. 2010)]; and (2) plasma membrane acid-sensing ion channels 1a (ASIC1a) (Carnally et al. 2010; Herrera et al. 2008), which results in ASIC1a-mediated Ca^{2+} currents inhibition and consequent intracellular Ca^{2+} accumulation (Herrera et al. 2008).

Taken together, while it is clear that Sig-1R modulates Ca^{2+} channels, the modulation, depending on the physiological context, can be direct or indirect, and facilitatory or inhibitory. This highlights the complexity and diversity in Sig-1R actions on Ca^{2+} signaling and even suggests that the Sig-1R can exert opposite effects depending on the brain regions or neuronal subtypes.

4.2 Voltage-Gated Na^+ Channels

Direct interaction and modulation of Na^+ channel functions by Sig-1R has been reported only recently (Fontanilla et al. 2009; Johannessen et al. 2009; Zhang et al. 2010) (Table 1). Sig-1R activation in mouse cardiac myocytes and fibroblast-like cell lines [e.g., COS-7 and human embryonic kidney cells (HEK 293)] by (+)-SKF10,047, (+)-pentazocine (Johannessen et al. 2009) or *N*,*N*-dimethyltryptamine (DMT, Sig-1R putative endogenous ligand) (Fontanilla et al. 2009) inhibits $Na_v1.5$-mediated currents without altering its biophysical properties (Johannessen et al. 2009) (Fig. 2). This effect was attenuated in cells from SIGMAR1 knock-out mice. Further analysis showed that Sig-1R inhibits $Na_v1.5$-mediated currents without requiring ATP or GTP (Johannessen et al. 2009), suggesting G-protein- and protein kinase-independent mechanisms. AFM imaging of co-isolated Sig-1R/$Na_v1.5$ protein complexes provided unequivocal evidence for Sig-1R and $Na_v1.5$ protein–protein association, a process that also occurs in intact cells (Balasuriya et al. 2012) (Fig. 2, Table 1). Interestingly, only a small portion (6%) of the two proteins appeared to interact, suggesting that the Sig-1R may be involved in $Na_v1.5$ trafficking or maturation, a process that is reminiscent of Sig-1R-dependent regulation of K^+ channels (Crottes et al. 2011; Kinoshita et al. 2012; Kourrich et al. 2013). Functionally, although Johannessen et al. (2009) did not observe Sig-1R-mediated changes in Na^+ current biophysical properties (Johannessen et al. 2009), Zhang et al. (2010) found that Sig-1R ligand activation shifted steady-state inactivation of Na^+ channels to more negative potentials, which resulted in delayed action potential latency and decreased firing rate (Zhang et al. 2010).

In summary, Sig-1R has the capability to decrease Na^+ channel functions via two mechanisms, decreasing Na^+ current and Na^+ channel availability when needed. Taken together, and in contrast to bidirectional action of Sig-1R on Ca^{2+} currents, only inhibitory actions of Sig-1R on Na^+ currents have been reported so far.

4.3 Voltage-Gated K^+ Channels

Sig-1R modulates various K^+ channels in both non-neuronal and neuronal cells. This modulation can involve either indirect mechanisms or direct protein–protein interactions [reviewed in (Kourrich et al. 2012)]. Among the seminal studies that linked Sig-1R to K^+ currents (Bartschat and Blaustein 1988; Kennedy and Henderson 1990; Wu et al. 1991), Morio et al. (1994) were among the first to suggest direct regulation of K^+ channels by Sig-Rs (Morio et al. 1994). Although Sig-R antagonists were not used, the study provided convincing pharmacological and electrophysiological evidence showing that Sig-R agonists block a tonic and outward K^+ conductance. Disrupting second messenger systems with pertussis toxin, cholera toxin, forskolin, phorbol-12,13-dibutyrate, and absence of GTP in the recording pipette did not prevent Sig-R-induced blockade of the K^+ current, suggesting direct coupling between Sig-Rs and the identified channel. Studies from early 2000s provided additional evidence, in which disrupting ATP- and GTP-dependent processes in rodent neurohypophysial nerve terminals (Lupardus et al. 2000) or in parasympathic intracardiac neurons (Zhang and Cuevas 2005) did not prevent Sig-1R agonists from attenuating various K^+ currents (delayed outward rectifier K^+ current, I_{KDR}; large conductance Ca^{2+}-sensitive K^+ channels, I_{BK}; and M-current). Although Sig-1R agonists were used at high concentrations (up to 100 μM), which can lead to unspecific and direct pharmacological blockade of K^+ channels (Lamy et al. 2010; Lupardus et al. 2000), data showed that when Sig-1Rs agonists were applied on *Xenopus* oocytes that do not express Sig-1R, K^+ channels were unresponsive, ruling out inhibition through direct drug interaction with these K^+ channels. Nonetheless, the evidence was still indirect.

Today, unequivocal evidence for direct physical interaction between Sig-1Rs and K^+ channels is accumulating (Aydar et al. 2002; Crottes et al. 2011; Kinoshita et al. 2012; Kourrich et al. 2013; Balasuriya et al. 2014) (Fig. 2, Table 1). Regarding the Kv family, and particularly the Kv1 subfamily, a seminal study (Aydar et al. 2002), combining electrophysiological recordings and co-immunoprecipitation data from posterior pituitary gland and *Xenopus* oocytes, showed that the Sig-1R inhibits K^+ currents formed by Kv1.4 subunits through a direct protein–protein interaction, and consistent with co-localization studies in CHO-K1 cells (Mavlyutov and Ruoho 2007). Interestingly, Sig-1R differentially inhibited Kv1.4-mediated current through ligand-dependent or independent mechanisms, a functional relationship suggesting that the Sig-1R may act as a ligand-regulated auxiliary K^+ channel subunit. Similar to these findings, co-expression in *Xenopus* oocytes of Sig-1Rs and Kv1.3 channels, a slowly inactivating outward voltage-gated K^+ channel that is predominantly expressed in T lymphocytes (Panyi et al. 2004) and in cerebellum (Vacher et al. 2008), resulted in Kv1.3-mediated currents inhibition by accelerating channel inactivation in ligand-independent manner (Kinoshita et al. 2012). However, most of these studies were performed using reductionist models (e.g., heterologous expression system in cell cultures and Xenopus oocytes; and neuroendocrinal tissue in vitro), and it is only recently that Sig-1Rs have been found to bind to Kv channels in the brain and that external

stimuli boost the formation of these complexes, which results in long-lasting changes in both neuronal excitability and behavior (Kourrich et al. 2013). In brief, via protein–protein association Sig-1Rs contribute to basal trafficking of Kv1.2 channels in the nucleus accumbens (NAc, brain region involved in reward and motivation), the prefrontal cortex (PFC, brain region involved in decision-making), and in heterologous expression system (NG108-15 and Neuro2A cell lines), suggesting that the association between Kv1.2 and Sig-1Rs is a conserved mechanism. This study also provided both physiological and behavioral relevance to such functions of the Sig-1R in the intact animal. Specifically, systemic in vivo cocaine injections upregulate Kv1.2-mediated current in NAc neurons, a mechanism that is caused by enhanced and persistent protein–protein association between Sig-1Rs and Kv1.2 channels at the membrane. This adaptation was correlated to locomotor response to cocaine and was maintained long after cessation of cocaine administration (up to 14 days), which demonstrates that Sig-1R binding to K^+ channels can undergo enduring experience-driven plasticity that has direct consequences on behavior.

Sig-1R has been reported to be overexpressed in cancer cell lines (Crottes et al. 2013) where, via direct physical interaction (Balasuriya et al. 2014; Crottes et al. 2011), it functions as a chaperone enhancing maturation and membrane expression of the human ether-à-gogo K^+ channel (hERG, also known as Kv11.1), a voltage-dependent K^+ channel that regulates cardiac repolarization (Sanguinetti et al. 1995; Trudeau et al. 1995).

In summary, Sig-1R bidirectionally modulates K^+ currents, an effect that occurs through direct protein–protein interaction and resulting either in modulation of K^+ channels functions (Aydar et al. 2002; Kinoshita et al. 2012) or regulation of subunit maturation and trafficking to the cell surface (Crottes et al. 2011; Balasuriya et al. 2014; Kourrich et al. 2013).

5 Overall Neuronal Excitability

5.1 Is Sig-1R Critical for the Regulation of Basal Neuronal Excitability?

Because Sig-1R modulates so many client proteins, it is surprising that knocking out Sig-1R does not lead to more dramatic effects on behavior and neuronal activity. It is even more striking that knock-out of classical auxiliary subunits that regulate only a limited number of VGICs leads to profound changes in various cellular functions (Giese et al. 1998; Pongs et al. 1999; Martinez-Espinosa et al. 2014; Sun et al. 2011), and thereby leads to diseases such as epilepsy (Heilstedt et al. 2001), long QT syndrome (Schulze-Bahr et al. 1997; Splawski et al. 1997), muscle-related disorders (e.g., familial periodic paralysis, disorder of skeletal muscle) (Abbott and Goldstein 2001), and in some cases to premature death (Arikkath and Campbell 2003). However, based on the absence of both clear behavioral and physiological phenotypes observed in naïve Sig-1R KO mice

(Langa et al. 2003), it becomes legitimate to wonder whether the Sig-1R is critical for the regulation of basal neuronal activity and behavior. One might hypothesize that Sig-1R function becomes critical only when the system is challenged. This point can be supported by both behavioral and cellular evidence.

For example and regarding behavior, while Sig-1R knockout models do not exhibit notable changes in basal behavior, animals exhibit impairments when they must learn and memorize new information (Entrena et al. 2009; Chevallier et al. 2011), having to cope with depression (Chevallier et al. 2011; Sabino et al. 2009), or having to assess pain levels (Cendan et al. 2005). To further support this hypothesis, activation of the Sig-1R plays a protective role when the system is challenged as reported for several disorders of the nervous system or affects, including amnesia, depression, neuropathic pain, Alzheimer's disease, and stroke (Maurice and Su 2009). Altogether, the Sig-1R may have little role to play in healthy systems and would play a beneficial role only upon dysregulations of cellular functions. An exception to this hypothesis is the consensus in the field that Sig-1R activation contributes to the development of drug addiction (Katz et al. 2011) and cancer (Crottes et al. 2013).

To date, at the cellular excitability level, application of Sig-1R antagonists alone in various in vitro preparations or in intact animals has no effect on Ca^{2+} dynamics (Cuevas et al. 2011; Pan et al. 2014; Tchedre et al. 2008), K^+ currents (Kourrich et al. 2013), and Na^+ currents (Zhang et al. 2009). And shRNA-mediated Sig-1R knockdown in the NAc shell in vivo does not alter basal neuronal excitability either (Kourrich et al. 2013). However, it is noteworthy to mention that acute pharmacological blockade of Sig-1R cannot be compared with lasting protein knockdown conferred by shRNAs. Data indicate that shRNA-induced Sig-1R knockdown in cell culture preparations reduces hERG (Crottes et al. 2011) and $Na_v1.5$ current densities (Balasuriya et al. 2012).

Although the question of whether the Sig-1R is critical for the regulation of basal neuronal excitability remains open, in summary and as an analogy, the role of the Sig-1R could be compared to the police, where its role is minimal in safe environment but becomes critical when safety and order are compromised.

5.2 Net Effect of Sig-1R Activation on Overall Neuronal Excitability

The regulatory functions of the Sig-1R on various aspects that control neuronal activity are diverse, including the regulation of post-translational biogenesis of ion channels, their trafficking to the plasma membrane, ligand-independent modulation of VGIC functions, and modulation of neurotransmitter release. Contributions of these processes are often brain region- and cell type-specific, resulting in complex effects on neuronal excitability and thereby on brain circuits' function. What dictates the net effect of these Sig-1R-dependent modulations on mechanisms that control overall neuronal activity is one of today's challenges in the field of Sig-1R and neuronal excitability. The complexity and diversity of Sig-1R's targets

has been the main issue limiting the understanding of how Sig-1R functions affect CNS activity.

For example, inhibition of Na^+ currents by the Sig-1R (Johannessen et al. 2009; Zhang et al. 2009) should decrease action potential firing, whereas inhibition of K^+ currents (Aydar et al. 2002; Crottes et al. 2011; Kinoshita et al. 2012) should, in contrast, increase action potential firing. To add a supplementary level of complexity, depending on the neuroanatomical site of action, the Sig-1R facilitates (Sabeti et al. 2007) or inhibits (Tchedre et al. 2008) voltage-gated Ca^{2+} channels. And investigating the role of Sig-1R on neuronal intrinsic excitability is further challenged by the complexity and diversity of ion channel subtypes, e.g., over 100 genes have already been cloned for the pore-forming α subunits of K+ channels alone (Lujan 2010). Thus, it is expected that future studies will reveal novel ion channel targets of Sig-1R, and therefore, inferring the changes in firing capacity from changes in individual (or even several) currents may be misleading (Marder and Goaillard 2006). One way to approach this task is to measure firing properties before and after Sig-1R manipulation. Because of the factors discussed below, it is not surprising that the few studies that measured so far the effect of Sig-1R activation on basal firing have provided mixed results, showing both excitation and inhibition (Cheng et al. 2008; Zhang and Cuevas 2005; Zhang et al. 2009; Lucas et al. 2008; Ceci et al. 1988). Below are examples of biological factors that may explain the various neuronal responses to Sig-1R stimulation:

First, the availability of the targets is probably one of the most influential factors. Subtypes of Na^+, Ca^{2+}, and K^+ channels are heterogeneously and not proportionately distributed throughout the nervous system. Combined with variability in their subcellular distribution (Lujan 2010; Nusser 2009), these factors are likely to influence the resulting effect of Sig-1R activation on neuronal firing.

Second and reminiscent of the previous point, the intracellular milieu and molecular substrates available may also play important roles. Indeed, protein kinases [e.g., extracellular signal-regulated kinase (ERK)] and the Sig-1R can mutually regulate one another (Cormaci et al. 2007; Moriguchi et al. 2011). Protein kinases are strong modulators of VGICs (Cerda and Trimmer 2010). Therefore, variations in the types and levels of activity of protein kinases may lead to differential effects of Sig-1R activation on neuronal excitability. Further, a characteristic that defines neuronal types is the level of tonic activity, which suggests that modulatory effects mediated through the Sig-1R will also be influenced by neuronal and/or neural system basal activity.

Third, a factor that is often underestimated is age. Studies on age-dependent changes of Sig-1R levels have provided mixed results (Ishiwata et al. 2003; Phan et al. 2003); however, the levels of neuroactive steroids (important Sig-1R endogenous ligands) decrease with age (Maurice et al. 2001). As mentioned earlier, the action of neurosteroids on Sig-1Rs can affect both VGICs and LGICs, which indicates that endogenous age-related variations in neurosteroid levels could also contribute to physiological regulation of neuronal activity. This raises concerns when investigating the effects of Sig-1R stimulation on neuronal excitability using preparations from animals of different ages. In other words, exogenous

experimental conditions may interact with the endogenous level of neuroactive steroids and lead to different functional outcomes. For example, administration of the same concentration of neuroactive steroids to preparations from animals of different ages may lead to unexpected variability when neuronal excitability or synaptic transmission is investigated, an example that is well illustrated by the effects of PREGS on excitatory glutamate transmission (Zheng 2009). This factor may also lead to different behavioral effects when neurosteroids are administered in vivo.

Taken together, the net effect of Sig-1R activation on neuronal activity may depend on several factors, including the level of Sig-1R expression, availability of Sig-1R target proteins, anatomical and subcellular localization of VGICs, and the intracellular milieu, a factor that is influenced by the level of basal neuronal activity and age.

6 Concluding Remarks

In summary, Sig-1R, through various means and diverse targets, is capable of affecting each stage of neuronal transmission. This may explain why Sig-1R is associated with many brain functions and neurological disorders. A clear, region-specific understanding of how Sig-1Rs can regulate neuronal activity through the modulation of VGICs and LGICs will provide information not only on how Sig-1Rs participate in shaping neuronal activity, but also on how its disruption can lead to symptoms observed in brain disorders. However, one of the important challenges in the field is to establish causal relationships between specific Sig-1R-triggered signaling pathways, or specific Sig-1R-protein associations, and physiological outcomes. This advancement in basic biochemical and cellular mechanisms will contribute to the development of treatments that could target specific Sig-1R-associated diseases and disorders.

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Sigma-1 Receptor and Pain

Manuel Merlos, Luz Romero, Daniel Zamanillo, Carlos Plata-Salamán, and José Miguel Vela

Contents

Abstract

There is a critical need for new analgesics acting through new mechanisms of action, which could increase the efficacy respect to existing therapies and/or reduce their unwanted effects. Current preclinical evidence supports the modulatory role of the sigma-1 receptor (σ_1R) in nociception, mainly based on the pain-attenuated phenotype of σ_1R knockout mice and on the antinociceptive effect exerted by σ_1R

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antagonists on pain of different etiology, very consistently in neuropathic pain, but also in nociceptive, inflammatory, and visceral pain. σ_1R is highly expressed in different pain areas of the CNS and the periphery, particularly dorsal root ganglia (DRG), and interacts and modulates the functionality of different receptors and ion channels. Accordingly, antinociceptive effects of σ_1R antagonists both acting alone and in combination with other analgesics have been reported at both central and peripheral sites. At the central level, behavioral, electrophysiological, neurochemical, and molecular findings support a role for σ_1R antagonists in inhibiting augmented excitability secondary to sustained afferent input. Moreover, the involvement of σ_1R in mechanisms regulating pain at the periphery has been recently confirmed. Unlike opioids, σ_1R antagonists do not modify normal sensory mechanical and thermal sensitivity thresholds but they exert antihypersensitivity effects (antihyperalgesic and antiallodynic) in sensitizing conditions, enabling the reversal of nociceptive thresholds back to normal values. These are distinctive features allowing σ_1R antagonists to exert a modulatory effect specifically in pathophysiological conditions such as chronic pain.

Keywords
Allodynia • Analgesia • Antinociception • Chronic pain • E-52862 • Hyperalgesia • S1RA • Sigma-1 receptor

1 Introduction

Acute pain has evolved as a key physiological alert system for avoiding noxious stimuli and protecting damaged regions of the body by discouraging physical contact and movement (Jamieson et al. 2014). Conversely, chronic pain has been recognized as pain that persists beyond normal healing time and hence lacks the acute warning function of physiological nociception. Chronic pain, defined as pain lasting or recurring for more than 3–6 months, may be associated with many common diseases or considered a disease by itself. It can be debilitating, with those affected typically suffering psychological disturbance and significant activity restrictions. Chronic pain is a frequent condition, affecting an estimated 20% of people worldwide and accounting for 15%–20% of physician visits (Treede et al. 2015). Moreover, chronic pain is accompanied with other comorbidities, such as depression, deeply affecting patient's quality of life. Unfortunately, currently available treatments provide the modest improvements in pain and minimum improvements in physical and emotional functioning (Turk et al. 2011). Thus, the unmet medical need in the pain area is huge, and particularly relevant in difficult-to-treat pain modalities, such as neuropathic pain.

Despite massive efforts coming from basic science and clinical research, pain management remains a clinical challenge, with many patients still suffering with unrelieved or undertreated pain. There is a lack of real breakthrough innovation in the field (Kissin 2010; Labianca et al. 2012) and thus a need for new drugs acting through new mechanisms of action, which could increase the efficacy of existing therapies and/or reduce their unwanted effects.

The sigma-1 receptor (σ_1R), a unique ligand-regulated chaperone protein with no precedent and no homology to known proteins (Almansa and Vela 2014), has become one among the new and most promising pharmacological targets in pain. σ_1R was found to be unique, with no significant similarity with any other known mammalian protein receptors, and to have about 90% amino acid identity and 95% similarity across species (Hanner et al. 1996; Kekuda et al. 1996; Seth et al. 1997). From a functional point of view, the σ_1R physically interacts with a variety of receptors and ion channels or elements of their transduction machinery and acts as a modulator of their activity. At the endoplasmic reticulum, the σ_1R acts as a ligand-operated molecular chaperone regulating Ca^{2+} flow via inositol 1,4,5-trisphosphate (IP_3) receptors (Hayashi and Su 2007; Su et al. 2010). σ_1Rs, through their molecular chaperone activity, regulate protein folding/degradation, oxidative stress, and cell survival (see Hayashi (2015) for a review). In the plasma membrane, σ_1R interacts with components of the plasma membrane-bound signal transduction to modulate the activity of neurotransmitter receptors and ion channels, including K^+ channels, Ca^{2+} channels, *N*-methyl-D-aspartate receptor (NMDAR), and opioid receptors (see Zamanillo et al. (2013) for a review). Interestingly, its activity can be modulated (enhanced or inhibited) by σ_1R ligands in an agonist–antagonist manner.

The purpose of this review is to summarize the current knowledge on the involvement of σ_1R in pain modulation. First, regarding the site of action, the role of σ_1R in central sensitization phenomena has been reported at the behavioral, electrophysiological, neurochemical, and molecular levels. In contrast, the involvement of σ_1R in mechanisms regulating pain at the periphery has been recently confirmed and requires further investigation. Second, due to the chaperoning activity of the σ_1R, the current understanding of its interaction with different other molecular targets involved in pain transduction, transmission, and processing is summarized. Third, we have addressed the role of σ_1R in pain gathering at the experimental level using genetic approaches, i.e., by the use of σ_1R knockout (KO) mice or antisense probes, as well as pharmacological tools, including nonselective marketed drugs and experimental drugs in discovery and clinical development phases. The use of σ_1R KO mice has been critical to identify the σ_1R as a modulator of activity-induced sensitization of pain pathways. Accordingly, σ_1R KO mice are insensitive or show attenuated expression of pain behaviors in chemically induced (e.g., formalin and capsaicin) and neuropathic pain models (Cendan et al. 2005b; Entrena et al. 2009b; de la Puente et al. 2009; Nieto et al. 2012, 2014; Gonzalez-Cano et al. 2013; Gris et al. 2014; Tejada et al. 2014). These genetic as well as pharmacological findings using several σ_1R ligands (see Vela et al. (2015) for a review) provided evidence to consider σ_1R antagonists as an innovative and alternative approach for treating pain, especially neuropathic pain but also other sensitizing pain conditions. Notwithstanding the foregoing, several discrepancies between the information coming from the σ_1R KO mice and pharmacological approaches have been reported and the possible causes are discussed.

Preclinical evidence has pointed out their potential as an adjuvant therapy to enhance opioid analgesia, without increasing the side effects associated with opioid use (Chien and Pasternak 1994; Vidal-Torres et al. 2013; Sanchez-Fernandez et al. 2013, 2014). The modulation of opioid system by the σ_1R is fully covered in another

chapter of this book. As an advantage over opioids, σ_1R antagonists do not alter normal basic pain behavior as they do not modify the normal sensory mechanical and thermal perception in the absence of sensitizing stimuli. That is, σ_1R antagonists exert antiallodynic and antihyperalgesic effects in sensitizing conditions, enabling the reversal of diminished nociceptive thresholds back to normal values, but they do not modify normal sensory thresholds in non-sensitizing conditions, i.e., in normal conditions, in the absence of injury or other inductors of pain hypersensitivity (Chien and Pasternak 1995; Kim et al. 2008; Entrena et al. 2009b; Romero et al. 2012). Among the σ_1R antagonists, E-52862 (also known as S1RA) is the leading compound in the field and the only currently being developed for the treatment of pain. It was identified in a medicinal chemistry program as a highly active and selective σ_1R antagonist (Diaz et al. 2012). It was safe, well-tolerated, and showed good pharmacokinetic profile following oral administration to human volunteers in phase I studies (Abadias et al. 2013) and it is currently undergoing Phase II clinical trials for the treatment of different types of pain.

2 Localization of σ_1R in Relation to Pain Transmission: Central Vs Peripheral

σ_1R is expressed in several areas of the CNS specialized in nociceptive signaling processing, including the dorsal horn (DH) of the spinal dorsal cord, thalamus, periaqueductal gray (PAG), basolateral amygdala, and rostroventral medulla (RVM) (Alonso et al. 2000; Phan et al. 2005). σ_1R is also expressed in peripheral dorsal root ganglia (DRG) neurons (Guitart et al. 2004; Bangaru et al. 2013). Importantly, its high density in DRG, in which σ_1R expression is roughly an order of magnitude higher than in several CNS areas involved in pain signaling, points to a functional role of peripheral σ_1R in pain modulation (Sanchez-Fernandez et al. 2014). σ_1R is expressed by both sensory neurons and satellite cells in rat DRGs and its expression is regulated in axotomized neurons and in accompanying satellite glial cells (Bangaru et al. 2013). In accordance with σ_1R anatomical distribution, the antinociceptive effects of σ_1R antagonists both when acting alone and in combination with opioids to enhance opioid analgesia have been reported at both central and peripheral sites. A systematic review of σ_1R-dependent central and peripheral mechanisms in pain processing and development can be found in Romero et al. (2016).

2.1 Effect of σ_1R Antagonists at Central Sites: Inhibitory Effect on Central Sensitization

Central sensitization is responsible for many of the temporal, spatial, and threshold changes in pain sensibility and exemplifies the fundamental contribution of the CNS to the generation of pain hypersensitivity. Central sensitization results from changes in the properties of neurons in the CNS. Thus, pain is no longer coupled to the presence, intensity, or duration of noxious peripheral stimuli as it occurs in acute nociceptive

pain. Instead, central sensitization produces pain hypersensitivity by exaggerating the sensory response elicited by nociceptive suprathreshold stimuli and allowing the response to subthreshold stimuli, including those that usually evoke innocuous sensations (D'Mello and Dickenson 2008).

An inhibitory effect has been attributed to σ_1R antagonism on central sensitization phenomena, as supported at the behavioral (animal pain models), electrophysiological (spinal wind-up recordings), neurochemical (spinal release of neurotransmitters), and molecular (NMDAR function regulation) levels. Activation of primary afferent nociceptive fibers subsequent to intradermal injection of some chemical irritants, including capsaicin or formalin, into the plantar skin of the hind paw in rodents or into the skin of humans produces acute/immediate nociceptive behaviors followed by long-lasting, secondary mechanical hypersensitivity (e.g., mechanical allodynia) that results from central sensitization (O'Neill et al. 2012). Interestingly, capsaicin was unable to induce mechanical hypersensitivity in σ_1R KO mice, and the effect in σ_1R KO mice was mimicked in wild-type (WT) animals treated with BD1063, BD1047, or NE100, three σ_1R antagonists which dose-dependently inhibited capsaicin-induced mechanical allodynia (Entrena et al. 2009b). Other σ_1R antagonists including haloperidol and its metabolites I and II (Entrena et al. 2009a), E-52862 (Romero et al. 2012) and some spirocyclic thiophene bioisosteres (Oberdorf et al. 2008), 1′-benzyl-3-methoxy-3H-spiro[[2]benzofuran-1,4′-piperidine] (Wiese et al. 2009), and a 1,3-dioxane ligand 2 (Utech et al. 2011) also produced antiallodynic effects in the capsaicin model. In addition, the σ_1R agonist PRE-084 reversed the effect of antagonists (Entrena et al. 2009a, b), further supporting the role played by σ_1R in capsaicin-induced central sensitization phenomena. In the formalin-induced pain model in mice, both phases of pain were reduced by approximately 55% in mice lacking σ_1R in comparison to WT animals (Cendan et al. 2005b). Shortly after this study, the same authors reported that haloperidol and its metabolites I and II, which have affinity for σ_1R, dose-dependently inhibited formalin-induced pain in mice through a mechanism likely involving antagonism on σ_1R (Cendan et al. 2005a). Subsequent studies using selective and prototypical σ_1R antagonists such as E-52862 (Romero et al. 2012; Vidal-Torres et al. 2014) and BD1047 (Kim et al. 2006), and novel σ_1R antagonists based on pyrimidine (Lan et al. 2014a) or 3,4-dihydro-2(1H)-quinolinone (Lan et al. 2014b) scaffolds corroborated these initial findings and pointed to the spinal cord and supraspinal CNS regions as sites for the σ_1R-mediated modulation of formalin sensitization. The spinal cord was first pointed out in the study by Kim et al. in mice, where intrathecal (i.t.) pretreatment with the σ_1R antagonist BD1047 dose-dependently reduced formalin-induced pain behaviors in the second phase, but not in the first phase of the formalin test, concomitant with reduced formalin-evoked Fos expression in spinal DH neurons (Kim et al. 2006). In addition to the spinal cord, supraspinal sites were supported by the finding in rats that i.t. pretreatment with E-52862 attenuated the formalin-induced flinching behavior, but not lifting/licking behaviors, whereas E-52862 also attenuated lifting/licking when intracerebroventricularly (i.c.v.) injected (Vidal-Torres et al. 2014). In this way, it is interesting to note that flinching is a spinal response whereas lifting/licking behaviors are supraspinal responses (Coderre et al. 1994), and that both spinal and supraspinal descending modulation of central neural plasticity occur in formalin-induced pain (Coderre et al. 1994;

Vaccarino and Chorney 1994). Therefore, it is concluded that σ_1R acts in the CNS at both spinal and supraspinal sites to modulate pain sensitization following sustained peripheral activation of nociceptors by formalin.

2.2 Effect of σ_1R Antagonists at Peripheral Sites: Inhibitory Effect on Peripheral Sensitization

Increasing evidence suggests that activity from the periphery is essential, not only to initiate but also to maintain pain (Richards and McMahon 2013). Experience from clinical studies using lidocaine and capsaicin patches, local steroids, and regional anesthesia, among others, clearly demonstrates that blocking the peripheral nociceptive input is an effective strategy to relieve chronic pain. Studies focused on finding new analgesic strategies with a peripheral site of action merit further efforts, as targeting the periphery could be a good approach to overcome the typical side effects related to CNS actions of current analgesics.

Although the role of peripheral σ_1R in pain has not been extensively studied (Tejada et al. 2014), recent pieces of information are actually confirming their involvement in mechanisms regulating pain, both when administered alone or in combination with opioids. Systemic administration of the selective σ_1R antagonist E-52862 produced an attenuation of the flinching and lifting/licking behaviors in the formalin test in rats, which was concomitant with an enhancement of noradrenaline levels and a reduction of formalin-evoked glutamate release in the spinal DH. Although a supraspinal effect was confirmed by the local (i.c.v) administration of E-52862, a peripheral contribution was also shown. In fact, intraplantar (i.pl.) administration of E-52862 in the ipsilateral paw (but not in the contralateral) reduced lifting/licking behaviors in phase I and II of the formalin test (Vidal-Torres et al. 2014).

Recent studies have also evaluated the role of σ_1R in inflammatory pain (Gris et al. 2014; Parenti et al. 2014; Tejada et al. 2014; for review see Gris et al. (2015)). Systemic administration of several σ_1R antagonists was effective in the carrageenan- and complete Freund adjuvant-induced pain models. Particularly, the study by Tejada et al. described the importance of peripheral σ_1R in the carrageenan-induced pain model in mice. The local (i.pl.) administration of the σ_1R agonist PRE-084 abolished the systemic antihypersensitive effect of the σ_1R antagonists BD1063 and E-52862. Moreover, the i.pl. administration of the σ_1R antagonist E-52862 in the inflamed paw was sufficient to completely reverse inflammatory hyperalgesia. The antihyperalgesic effect of locally administered E-52862 was reverted by the i.pl. administration of the σ_1R agonist PRE-084 and was absent in σ_1R KO mice, thus confirming that the peripheral antihyperalgesic effect of E-52862 was mediated through σ_1R. As a conclusion, a number of reports have revealed the possibility of targeting peripheral σ_1R to ameliorate inflammatory hyperalgesia (Gris et al. 2015; Tejada et al. 2014). A peripheral σ_1R-related mechanism might be more relevant in the modulation of inflammatory pain than in pain evoked by other etiologies because this type of pain is characterized by a pronounced enhancement of nociceptor responsiveness (peripheral sensitization) in response to the inflammatory mediators released at the inflammation site (Xu and

Yaksh 2011). Due to its pleiotropic chaperoning nature and acting downstream to the activation of different receptors and channels, σ_1R could modulate the intracellular signaling of a variety of pro-algesic mediators released at the inflamed site. Among them, bradykinin and nitric oxide (NO) are key mediators released during inflammation contributing to peripheral sensitization (Wang et al. 2006; Petho and Reeh 2012). σ_1R activation enhances both bradykinin-induced Ca^{2+} signaling in neuronal-like cell cultures (Hayashi et al. 2000) and NO signaling (Roh et al. 2011). In addition, pain sensitization after peripheral inflammation involves plastic changes mediated by an increase in spinal excitatory neurotransmission together with activation of kinases, including ERK1/2, which are known to be modulated by σ_1R (de la Puente et al. 2009).

In addition to inflammatory pain, the contribution of peripheral σ_1R to ischemic pain has been recently demonstrated in a rat model of hind limb thrombus-induced mechanical allodynia (Kwon et al. 2016). σ_1R expression significantly increased in skin, sciatic nerve, and DRG at 3 days post-thrombus-induced ischemic pain in rats. Authors suggested a facilitating effect of σ_1R on acid-sensing ion channels (ASICs) and purinergic P2X receptors, as i.pl. injection of the σ_1R antagonist BD1047 reduced mechanical allodynia synergistically with the ASIC blocker amiloride and the P2X antagonist TNP-ATP (Kwon et al. 2016). Regarding neuropathic pain, σ_1R antagonism has been shown to restore injury-induced decrease in voltage-gated Ca^{2+} current in dissociated rat DRG neurons following spinal nerve ligation but had no effect on control and non-injured DRGs, which is discussed as an antinociceptive mechanism as inward Ca^{2+} currents are required for natural suppression of repetitive firing via opening of Ca^{2+}-activated K^+ channels (Pan et al. 2014).

3 Neuroprotective Effects of σ_1R Antagonists in Relation to Pain

Neuroprotective but also neurotoxic roles have been attributed to σ_1R in the CNS by mechanisms involving modulation of cellular Ca^{2+} homeostasis, excitotoxicity, oxidative and nitrosative damage, and endoplasmic reticulum and mitochondrial stress. Indeed, both σ_1R agonists (DeCoster et al. 1995; Shimazu et al. 2000; Vagnerova et al. 2006; Mancuso et al. 2012; Griesmaier et al. 2012) and antagonists (DeCoster et al. 1995; Shimazu et al. 2000; Schetz et al. 2007; Luedtke et al. 2012) have been reported to exert protective effects on neurons using different in vitro and/or in vivo experimental approaches. In the context of pain, it has been reported that σ_1R antagonism exerts a preventive effect against peripheral neuropathy. In particular, genetic inactivation (σ_1R KO mice) and pharmacological blockade of σ_1R prevented paclitaxel-induced sensory nerve mitochondrial abnormalities, concomitant with the prevention of paclitaxel-induced cold and mechanical allodynia (Nieto et al. 2014). In contrast, the σ_1R agonist SA4503, but not the σ_1R antagonist NE100, produced antinociceptive effects against chemotherapeutic-induced neuropathic pain in rats (Tomohisa et al. 2015). Mitochondrial function/dysfunction has been suggested as a causal or contributory mechanism of normal sensory processing and chronic pain, not only in painful peripheral neuropathies evoked by chemotherapy but also in diabetes and HIV (Flatters 2015). σ_1Rs at the

endoplasmic reticulum–mitochondrion contact are known to regulate mitochondrial function, including intramitochondrial Ca^{2+} homeostasis, oxidative stress, and cellular bioenergetics (Su et al. 2010; Hayashi 2015). The role played by σ_1R in regulating pain-related mitochondrial dysfunction merits further investigation.

4 σ_1R Pain Interactome

The σ_1R, as a ligand-operated chaperone, is able to interact with other proteins including receptors, enzymes, or ion channels, many of which are involved in nociception. Figure 1 shows the known regions of the σ_1R that are involved in its direct interaction with other protein partners. In the receptor's N terminal part, there appears to be the interaction motifs for the NMDAR NR1 subunit, as well as the small five-amino acid dimerization motif for the σ_1R (Rodriguez-Munoz et al. 2015). Whether other interacting partners, like, for instance, ion channels described to interact with the σ_1R, do use this N terminal part of the receptor is still unknown. Interestingly, in the other part of the receptor, the C terminal part comprising from the transmembrane domain, several proteins share the same interaction region with the σ_1R (Ortega-Roldan et al. 2013; Su et al. 2016). These proteins are the ankyrin B, BiP, and IP_3 receptors that along with the σ_1R play important roles in endoplasmic reticulum Ca^{2+} homeostasis. Also, as explained above, while in control situations the σ_1R interacts mainly with ankyrin B and BiP, a reduction of these interactions and an increase in the interaction with IP_3 receptors are observed following a pathological stress insult (Su et al. 2016). These data point out that ankyrin B, BiP, and IP_3 receptors are competing each other for their binding with the σ_1R and that some interactions prevail over the others depending on the surrounding intracellular environmental conditions. Also, although the interaction region with these proteins is relatively large and the particular amino acids involved in each of them are not completely known, the fact that this competition exists between them suggests that at least part of those interaction regions must be shared. Interestingly, this competition is regulated as well by σ_1R ligands. New information regarding the interaction motifs of σ_1R partners to know better which regions of the receptor are responsible for chaperone activity and which partners can interact simultaneously or through a competitive manner is needed.

Pain is a very complex pathological condition and either nociceptive or neurogenic pain involves various interactive mechanisms at different neuronal levels such as peripheral nociceptors, spinal cord, or supraspinal levels. At all those levels, many chemical mediators and their molecular targets are engaged to code for and transmit the pain sensation (Millan 1999). σ_1R, playing its role as a chaperone protein, has been implicated in the regulation of many of those other molecular targets, including receptors, enzymes, and ion channels that are involved in pain sensation and transmission. Our objective in this section is to summarize σ_1R molecular partners, linking the regulation of these interactions to nociception, and thus describing the σ_1R pain interactome.

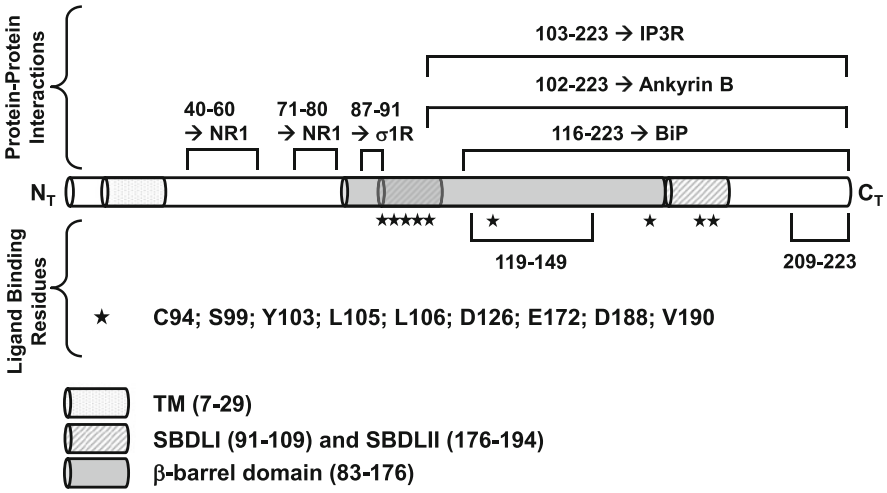

Fig. 1 Diagram of known sigma-1 receptor (σ_1R) motifs involved for interaction with their molecular partners and residues involved in ligand recognition. Protein–protein interaction domains are represented above the σ_1R sequence diagram, while the residues involved in ligand recognition are represented below. Regions of the σ_1R that mediate the interaction with the NR1 subunit of NMDA receptors and its oligomerization are comprised in the N terminal part of the receptor delimited till the first sterol binding domain. The C terminal part of the σ_1R starting from the first sterol binding domain is a region of the receptor clearly involved in its chaperone role as it serves to interact with proteins such as the IP_3 receptors, BiP, and ankyrin B. The recently described unique transmembrane domain (TM) and the β-barrel domain, which is involved in ligand-binding, and both steroid binding domains (SBDLs) are also depicted (Schmidt et al. 2016). Studies involving deletion of the 119–149 or 209–223 amino acid regions, mutations, or photolabeling of C94, S99, Y103, L105, L106, D126, E172, D188, and V190 have implicated all these amino acids in ligand recognition

4.1 Ion Channels

4.1.1 Voltage-Gated Sodium Channels

Nociceptors detect noxious stimuli and transmit this sensation to the CNS by means of action potentials. The fast upstroke of the action potential is generated through sodium channel activation (Liu and Wood 2011). A direct interaction of σ_1R with neuronal sodium channels has not been described yet, but σ_1Rs have been shown to co-immunoprecipitate with Nav1.5, the cardiac sodium channel, when transfected in tsA201 cells (Balasuriya et al. 2012). Both the nonselective σ_1R antagonist haloperidol and the σ_1R agonist (+)-pentazocine have been described to disrupt the $Na_v1.5$/σ_1R interaction, haloperidol being more effective in reducing this interaction (Balasuriya et al. 2012). Accordingly, independent on the agonistic or antagonistic nature of ligands, σ_1R agonists (+)-SKF-10047 and (+)-pentazocine and nonselective σ_1R/σ_2R ligands including haloperidol (antagonist) and 1,3-di-o-tolyl-guanidine (DTG) (agonist) all reversibly inhibited $Na_v1.5$ channels to varying degrees in HEK-293, COS-7 cells, and neonatal mouse cardiac myocytes (Johannessen et al. 2009). Patch-clamp

recordings in HEK293 cells stably expressing the human cardiac $Na_v1.5$ also revealed inhibitory modulation by some σR ligands, such as (+)-SKF-10047 and dimethyltryptamine (DMT), which was reverted by progesterone to varying degrees, consistent with antagonism of σ_1 and/or σ_2 receptors, and in some cases by σ_1R knockdown with small interfering RNA (Johannessen et al. 2011). Similarly, patch-clamp experiments in isolated intracardiac neurons from neonatal rats revealed that the nonselective σ_1R/σ_2R agonist DTG and the σ_1R selective agonist (+)-pentazocine inhibited voltage-gated sodium channels. The selective σ_1R antagonist BD1063 did not modulate the current but inhibited DTG block of sodium currents by ~50%, suggesting that the effects involve, at least in part, σ_1Rs (Zhang et al. 2009). Action potential generation through very fast inactivating sodium current is followed by a non-inactivating or persistent current that normally comprises about 5% of the whole sodium current generated. This persistent sodium current has been involved in the setting of the membrane resting potential in a subthreshold range regulating repetitive firing and enhancing synaptic transmission (Kiss 2008). $Na_v1.8$ is a tetrodotoxin-resistant voltage-gated sodium ion channel that is expressed specifically in the DRG, in small-diameter unmyelinated sensory neurons, and is involved in nociception. It has been described that human $Na_v1.8$ channel displays slower inactivation kinetics and a larger persistent current than already described for this channel in other species (Han et al. 2015). It is tempting to speculate that the interaction of σ_1R described for the $Na_v1.5$ could as well apply for other sodium channels involved in pain, such as $Na_v1.8$ channels, and that its regulation of persistent sodium current in neuronal areas involved in pain could explain part of its role in nociception. Nevertheless, studies investigating the relationship between σ_1R and sodium channels have been hampered by the lack of selectivity of several of the pharmacological tools utilized, thus precluding generalized conclusions. As an example, σ_1R agonists such as (+)-SKF-10047, dextromethorphan, and DTG have been found to directly inhibit $Na_v1.2$ and $Na_v1.4$ currents, apparently through a σ_1R-independent mechanism (Gao et al. 2012).

4.1.2 Voltage-Gated Potassium Channels

While sodium channels play a very prominent role in action potential generation producing depolarization, potassium counterparts play the opposite role leading to repolarization. The opening of potassium channels generates a hyperpolarizing potassium efflux across the membrane that counteracts inward ion conductance to limit neuronal excitability and firing rate (Tsantoulas and McMahon 2014). Not surprisingly, a role for potassium channels in nociceptive processing has been described (Tsantoulas and McMahon 2014). Cell lysates from nucleus accumbens medial shell tissue immunoprecipitated with specific $K_v1.2$ antibodies were shown to co-immunoprecipitate the σ_1R (Kourrich et al. 2013). This interaction was further confirmed in double transfected NG108-15 cells. $K_v1.2$ are delayed rectifier channels activated by slight membrane depolarization and are involved in the transient slowly inactivating potassium currents I_D. In CNS neurons, $K_v1.2$ channels are mainly localized at the axon initial segment where they modulate action potential threshold and firing rates, as well as nerve terminals where they control neurotransmitter release. In the peripheral nervous system, $K_v1.2$ are found in the soma and juxtaparanodes of medium-large DRG

neurons and are largely decreased after axotomy what may contribute to the hyperexcitable phenotype observed after such type of injury. Diminished $K_v1.2$ activity contributes to mechanical and cold neuropathic pain by depolarizing the resting membrane potential, reducing threshold current, and augmenting firing rates in myelinated neurons (Tsantoulas and McMahon 2014). Aydar and colleagues using co-immunoprecipitation techniques demonstrated a direct interaction with the $K_v1.4$ subtype in transfected xenopus oocytes and in rat posterior pituitary tissue. Not only a σ_1R agonist could elicit a decrease in $K_v1.4$ conductance in double transfected oocytes but also the expression of the σ_1R altered the functional activity of $K_v1.4$ expressed in these cells. In the presence of co-expressed σ_1R, $K_v1.4$ inactivated at a faster rate, and although net current efflux was also diminished, the voltage dependence of channel activation showed no change (Aydar et al. 2002). σ_1R agonists could elicit a decrease in $K_v1.4$ conductance in double transfected oocytes, but the co-expression of σ_1R with $K_v1.4$ resulted in a faster rate of channel inactivation, a reduction in net current efflux and no change in the channel voltage-dependence activation. This ligand independent regulation and the physical interaction with $K_v1.4$ suggest a function for σ_1R as auxiliary subunits for voltage-activated potassium channels (Kourrich et al. 2013). An important observation is that $K_v1.4$ channels are the only K_v1 α subtype expressed in small-diameter DRG neurons, meaning that this channel subtype is in charge of potassium conductance in Aδ and C nociceptor fibers (Rasband et al. 2001). The regulation of this subtype of potassium channel by σ_1R in this particular type of nociceptors is consistent with the regulatory role that σ_1R plays in pain modulation.

4.1.3 Voltage-Gated Calcium Channels

Voltage-gated calcium channels (VGCC) are other ion channels involved in neuronal action potentials that contribute to pain pathophysiology (Perret and Luo 2009). They are comprised of five different families, N, T, L, P/Q, and R-type, all of which are present at some extent at the central and peripheral nervous system playing a role in neurotransmitter release, membrane depolarization and hyperpolarization, enzyme activation and inactivation, and gene regulation (Perret and Luo 2009). Tchedre and colleagues, based on co-immunoprecipitation studies, proposed the interaction between the σ_1R and the L-type VGCC endogenously expressed in the RGC-5 retinal ganglion cell line (Tchedre et al. 2008). At the functional level, they found that the σ_1R agonist (+)-SKF-10047 inhibited potassium chloride-induced Ca^{2+} influx in the RGC-5 cell line and Ca^{2+} currents in rat cultured primary RGCs (Tchedre et al. 2008). Also in retinal ganglion cells, co-localization studies demonstrated that σ_1Rs and L-type VGCCs co-localized and calcium imaging studies showed that σ_1R agonists (+)-SKF10047 and (+)-pentazocine inhibited calcium ion influx through activated VGCCs (L-type). Antagonist treatment using BD1047 potentiated Ca^{2+} influx through activated VGCCs and abolished inhibitory effects of the σ_1R agonists (Mueller et al. 2013). Similar data were obtained using rat intracardiac and superior cervical ganglia neurons where sigma ligands could decrease peak Ca^{2+} channel currents of N, P/Q, and R-types (Zhang and Cuevas 2002). In addition to affecting a broad population of calcium channel types, σ_1R ligands altered the biophysical properties of these channels,

accelerating channel inactivation rate and shifting the voltage dependence of both steady-state inactivation and activation toward more negative potentials. Both σ_1R agonists and antagonists depressed Ca^{2+} channel currents, with a rank order of potency (haloperidol > ibogaine > (+)-pentazocine > DTG) consistent with the effects being mediated by σ_2R and not by σ_1R (Zhang and Cuevas 2002). A similar behavior has been described in dissociated rat DRG neurons, as σ_1R agonists (+)-pentazocine and DTG inhibited Ca^{2+} currents in patch-clamp experiments (Pan et al. 2014). The effect was ascribed to σ_1R activation as it was blocked by the σ_1R antagonists BD1063 or BD1047. Both (+)-pentazocine and DTG showed similar inhibitory effect on axotomized DRG neurons as they shifted the voltage-dependent activation and steady-state inactivation of VGCC to the left and accelerated VGCC inactivation rate in both control and axotomized DRG neurons. On the contrary, while the antagonist BD1063 had no effect by itself in normal non-injured DRGs, its application increased Ca^{2+} currents in axotomized ones (Pan et al. 2014). Pan and colleagues already noticed these paradoxical results, as σ_1R antagonists exert antinociceptive effects while σ_1R agonists are pronociceptive, and it is also known that painful nerve injury is accompanied by reduction of Ca^{2+} current in axotomized sensory neurons, which in turn results in elevated sensory neuron excitability. Similarly, it should be noted that Ca^{2+} current inhibition by compounds such as gabapentin or pregabalin is also an antinociceptive strategy. The complexity and heterogeneity of calcium channel signaling throughout neuronal regions involved in pain was argued in order to explain this apparent contradiction. While at the DH terminals, calcium channel activity controls neurotransmitter release and its blockade results in less neurotransmission and hence pain relief, calcium channel inhibition elsewhere (and particularly at the periphery) can result in inhibition of calcium-activated potassium channels that are in control of after-hyperpolarization, membrane excitability, and firing frequency, leading to an opposite final output. That is, lowered inward Ca^{2+} current has the dominant, overriding effect of decreasing outward current through calcium-activated potassium channels, thus reducing after-hyperpolarization and thereby increasing excitability. Antagonism of sensory neuron σ_1Rs at peripheral sites, including DRGs, may thus relieve pain by rescuing Ca^{2+} currents required for natural suppression of repetitive firing via opening of calcium-activated potassium channels.

4.1.4 Calcium-Activated Potassium Channels

Apart from voltage-sensitive potassium channels, σ_1R has been described to regulate non-voltage-dependent, small conductance (SK) calcium-activated potassium channels (Martina et al. 2007). SK potassium channels are non-voltage-sensitive, potassium selective, and activated by an increase in intracellular Ca^{2+} concentrations. SK channels activation, through the Ca^{2+} increases produced after action potentials, mediates membrane hyperpolarization, which limits firing frequency of repetitive action potentials (Vergara et al. 1998). Ca^{2+} entry after synaptic activation opens SK channels that act to limit the amplitude of synaptic potentials and reduce Ca^{2+} influx through NMDARs (Ngo-Anh et al. 2005). It has also been established that Ca^{2+} influx through NMDAR could open Ca^{2+}-activated K^+ channels in several systems. Using the σ_1R agonist (+)-pentazocine and patch-clamp whole-cell recordings in CA1 pyramidal cells of rat

hippocampus, potentiation of NMDAR-mediated responses was found to occur via inhibition of SK channels, that would normally reduce the amplitude of synaptic potentials reducing Ca^{2+} influx through NMDARs (Martina et al. 2007). Moreover, the enhanced NMDAR activity was translated into an increased synaptic plasticity as evidenced by a long-term potentiation effect (Martina et al. 2007). Another study also found that DTG inhibited SK channel in midbrain dopaminergic neurons and transiently transfected HEK-293 cells, but other σ_1R agonists such as carbetapentane, (+)-SKF-10047, and PRE-084 had no or little effect. The effect of DTG was not affected by high concentrations of the σ_1R antagonist BD1047, which argues against a coupling of σ_1Rs to SK channels and suggests that DTG directly blocks SK channels (Lamy et al. 2010). Thus, in the absence of further studies, it is difficult to know whether σ_1R actually regulates NMDAR via SK channels or if it is a ligand- or cell type-dependent finding.

4.1.5 Acid-Sensing Ion Channels

ASICs are cationic (sodium-permeable) channels activated by extracellular protons which are responsible for acid-evoked currents in neurons. They are involved in nociception but also in learning, memory, and in pathological conditions such as ischemic stroke (Osmakov et al. 2014). A direct interaction between σ_1R and ASIC using atomic force microscopy (AFM) in double transfected HEK cells has been described, which can be modulated by σ_1R ligands. The σ_1R antagonist haloperidol was able to reduce the ASIC1a/σ_1R binding about 50% (Carnally et al. 2010). Moreover, σ_1R/ASIC physical interaction has also functional consequences. Thus, σ_1R agonists decreased acid-induced ASIC1a currents and intracellular Ca^{2+} elevations in rat cortical neurons (Herrera et al. 2008), an effect ascribed to σ_1R engagement because the inhibitory effect was counteracted by σ_1R antagonists. In contrast, in ischemic pain induced by hindlimb thrombus, the σ_1R antagonist BD1047 reduced mechanical allodynia at the periphery synergistically with the ASIC blocker amiloride, whereas the σ_1R agonist PRE-084 induced mechanical allodynia when coadministered with an acidic pH solution, thus suggesting that σ_1R activation facilitates ASICs to promote pain (Kwon et al. 2016).

4.1.6 Ligand-Gated Calcium Channels

Ligand-gated calcium channels such as the glutamate NMDAR also interact with σ_1R. Increased Ca^{2+} influx through NMDAR and increased level of phosphorylation of these glutamate receptors have been reported following σ_1R activation (Monnet et al. 2003; Roh et al. 2008; Kim et al. 2008). This increase in the NMDAR phosphorylation state and activity is accompanied by enhanced pain behaviors. Recently, a direct physical interaction of the σ_1R with the C terminal of the NMDAR NR1 subunit has been described (Balasuriya et al. 2013; Sanchez-Blazquez et al. 2014b; Rodriguez-Munoz et al. 2015) both in vitro and in vivo using different technical approaches including bimolecular fluorescent complementation in double transfected CHO cells, in vitro pull-down assays, co-immunoprecipitation, or co-localization immunohistochemistry from PAG. This physical interaction also modulates the cross-talk between opioid analgesia and NMDAR activity (Pasternak et al. 1995; Garzon et al. 2012).

Garzon's group have shown how σ_1R antagonists are able to uncouple the σ_1R-NMDAR association while increasing opioid analgesia and reducing the development of opioid tolerance. All these evidences suggest a role of the σ_1R in the regulation of synaptic plasticity, as NMDAR has been described to mediate different forms of plasticity including long-term potentiation and central sensitization, phenomena linked to forms of pain facilitation such as hyperalgesia and allodynia (Sandkuhler 2000; Rygh et al. 2002).

4.2 G Protein-Coupled Receptors and Intracellular Second Messenger Machinery

Several G protein-coupled receptors (GPCRs), including targets clearly involved in pain modulation such as the cannabinoid CB_1 and μ-opioid (MOR) receptors, have been described as σ_1R partners (Kim et al. 2010; Sanchez-Blazquez et al. 2014a). σ_1R modulation of opioid receptors was initially described by Chien and Pasternak (1993, 1994) demonstrating that σ_1R antagonists potentiate opioid analgesia. At the in vitro level, Kim and colleagues demonstrated both a physical, by co-immunoprecipitation experiments, and a functional interaction between MOR and σ_1R in transfected HEK cells. The functional consequences of such an interaction were assessed by means of a GTPγS assay, antagonists increasing opioid efficacy by shifting the EC_{50} values of opioid-induced GTPγS binding by three- to tenfold to the left (Kim et al. 2010). A detailed review of the interaction between MOR and σ_1R is covered in another chapter of this book. Cannabinoid receptors also play a role in analgesia and they have been shown to be distributed both in peripheral and CNS regions important for pain transmission (Romero-Sandoval et al. 2015). Similarly to MOR, a physical interaction with σ_1R has been described for CB_1 receptors (Sanchez-Blazquez et al. 2014a). A functional in vivo relationship between these two receptors was demonstrated using the tail-flick test. The NMDAR increased its activity in σ_1R KO mice and it was no longer regulated by cannabinoids as in WT counterparts. Moreover, NMDAR antagonism in the σ_1R KO animals produced no effect on cannabinoid analgesia. Pharmacological intervention showed similar results, because antagonizing σ_1R prevented NMDAR antagonists from reducing CB1 receptor-induced analgesia. For both σ_1R-MOR-NMDAR and σ_1R-CB_1-NMDAR protein complexes, histidine triad nucleotide binding protein 1 (HINT1) has been shown to be another interacting partner. Inhibitors of HINT1 enzymatic activity have been described to enhance morphine-induced analgesia while reducing the development of opioid tolerance (Garzon et al. 2015). A direct physical interaction between this protein and the σ_1R has been shown recently (Sanchez-Blazquez et al. 2014a) and the coordinated interaction of HINT1 and σ_1R with NMDAR and its GPCRs partners is able to control the analgesia mediated through those GPCRs. Nociceptors are activated by diverse mediators, such as glutamate, bradykinin, and substance P, which act through GPCRs coupled to Gαq proteins. These Gαq proteins lead to the activation of the phospholipase C (PLC) cascade of intracellular second messengers leading to the release of Ca^{2+} from intracellular stores (Tappe-Theodor et al. 2012). The ability of σ_1R to modulate this pathway,

and so indirectly GPCRs coupled to the PLC-inositol triphosphate (IP_3)-calcium signaling cascade, represents another link to pain modulation. σ_1R activation has been also shown to stimulate PLC to produce diacylglycerol (DAG) and IP_3 (Morin-Surun et al. 1999), which in turn leads to the activation of IP_3 receptors and efflux of intracellular Ca^{2+} to the cytoplasm. There is growing evidence that σ_1R is an important player at the endoplasmic reticulum (ER) regulating Ca^{2+} homeostasis. In such a role, σ_1R interacts directly with ankyrin B, BiP, or IP_3 receptors (Hayashi and Su 2001, 2007; Shioda et al. 2012) and ultimately regulates intracellular Ca^{2+} mobilization from the ER to mitochondria in the mitochondria-associated ER membrane (MAM) (Shioda et al. 2012). σ_1R activation leads to a diminished interaction with ankyrin and BiP, an increase in its interaction with IP_3 receptor, and finally a stabilization of IP_3 receptors, thus facilitating Ca^{2+} efflux. σ_1R agonists caused the dissociation of ankyrin B and IP_3 receptors and this activity correlated with the ability of these ligands to potentiate intracellular Ca^{2+} mobilization induced by bradykinin. This increase in Ca^{2+} could be reversed by a σ_1R antagonist (Hayashi et al. 2000). Similarly, in CHO cells overexpressing a C terminal EYFP tagged σ_1R, agonists, such as (+)-pentazocine and PRE-084, caused very significant uncoupling of the σ_1R-BiP complex, whereas antagonists, such as NE100 or haloperidol, were not able to modify that complex at all.

5 Oligomerization

σ_1R interacts with itself (Pal et al. 2007; Mishra et al. 2015). A GXXXG motif is involved in the oligomerization process, as mutations of this σ_1R region reduced the number of receptors in higher oligomeric states and favored smaller oligomeric forms (Fig. 1) (Gromek et al. 2014). These higher order oligomers have been also demonstrated more recently by means of FRET spectrometry (Mishra et al. 2015). Moreover, only oligomeric and not the monomeric forms of σ_1R could bind the specific agonist (+)-pentazocine. Another finding by Gromek and colleagues was that ligand binding to σ_1R oligomers could prevent the formation of the monomer form, emphasizing the important role that σ_1R oligomers have on its pharmacology. Thus, pharmacological activity of σ_1R ligands, including their pro- or antinociceptive activities, could be at least in part consequence of their influence in regulating and/or interacting with σ_1R oligomeric states. Recently, a trimeric crystal structure with agonist and antagonist bound ligands (one ligand per monomer) has been described (Schmidt et al. 2016).

6 Changes in σ_1R Receptor Expression in Pain Conditions

As mentioned above, the σ_1R is expressed in areas important for pain control such as DRG neurons, DH spinal cord, thalamus, PAG, and RVM. It is expressed by both sensory neurons and satellite cells in rat DRG (Bangaru et al. 2013). Its expression in the spinal cord is upregulated during the induction phase of neuropathic

pain following sciatic nerve constriction (Roh et al. 2008; Moon et al. 2014; Son and Kwon 2010) and in the brain 10 weeks after the induction of diabetic neuropathy (Mardon et al. 1999). Moreover, σ_1R expression significantly increased in skin, sciatic nerve, and DRG at 3 days in a model of thrombus-induced pain in rats (Kwon et al. 2016). On the contrary, σ_1R expression has been reported to be reduced in spinal cords following chemotherapy (oxaliplatin and paclitaxel) treatment (Tomohisa et al. 2015) and in axotomized neurons and accompanying satellite glial cells following spinal nerve ligation in rats (Bangaru et al. 2013). Therefore, regulation of σ_1R expression in neuropathic pain does not provide a direct explanation for pain relief after σ_1R blockade but could instead represent an adaptive counteracting mechanism.

7 Electrophysiological Studies

Intrinsic DH neurons receive efferent nociceptive stimuli and are also responsible for sending the nociceptive input to supraspinal structures (Almeida et al. 2004). Repetitive stimulation of the dorsal root at stimulus intensities activating nociceptive fibers, but not non-nociceptive sensory fibers, produces an amplification of the nociceptive signals in the spinal cord known as wind-up response. Wind-up is a short-term, frequency-dependent, amplification mechanism distinct from long-term potentiation, central sensitization, and pain hypersensitivity/hyperalgesia, but it is a form of homosynaptic central facilitation of nociceptive messages and a correlate of such phenomena (Dickenson and Sullivan 1987; Herrero et al. 2000). Pharmacological σ_1R antagonism modulates spinal excitability, as shown in isolated mice spinal cords superfused with the σ_1R antagonist E-52862 and stimulated electrically. E-52862 did not modify the Aβ-fiber-mediated non-nociceptive signaling and the response to single stimuli at C-fiber intensity, which is consistent with the behavioral observation that σ_1R antagonists did not alter the normal perception of sensory subthreshold and nociceptive suprathreshold inputs in non-sensitizing conditions (Cendan et al. 2005a; Entrena et al. 2009a; Romero et al. 2012). However, E-52862 dose-dependently inhibited the spinal wind-up phenomenon when repetitive stimulation of nociceptive afferent C-fibers was applied (Romero et al. 2012; Mazo et al. 2015). Accordingly, spinal wind-up amplification of the nociceptive signals was highly reduced in spinal cords from σ_1R KO compared to WT mice (de la Puente et al. 2009). Hence, electrophysiological data point to a modulatory role of σ_1R on spinal excitability, whereby pharmacological antagonism or the absence of the receptor in KO mice inhibits the amplified spinal response that would normally arise from repetitive nociceptor stimulation. Inhibition of spinal hyperexcitability could underlie the effects exerted by σ_1R antagonists on a wide variety of pain conditions in which sustained, repetitive afferent drive following injection of some chemical irritants (e.g., capsaicin and formalin), tissue injury/inflammation, or nerve damage comes to the spinal cord.

8 Neurochemical Studies

The spinal cord is an important gateway for peripheral pain signals transmitted to the brain. In chronic pain states, painful stimuli trigger afferent fibers in the DH to release neuropeptides and neurotransmitters, including excitatory (e.g., glutamate) and inhibitory (e.g., GABA) neurotransmitters (Thomas Cheng 2010). Modulation by σ_1R of formalin-evoked changes in neurotransmitter levels in the spinal DH was investigated using concentric microdialysis in the ipsilateral DH of awake, freely moving rats (Vidal-Torres et al. 2014). Levels of three key neurotransmitters were measured as a neurochemical correlate of three major neuronal components regulating DH neurons and accounting for spinal sensitization: glutamate for primary activating afferent inputs to the DH, GABA for local inhibitory DH interneurons, and noradrenaline for supraspinal descending inhibitory modulation of the DH. Formalin-induced nociception enhanced glutamate levels in the DH spinal cord, which is coherent with the activation of afferent glutamatergic nociceptive fibers. Systemic administration of the σ_1R antagonist E-52862 exerted antinociceptive effects on formalin-induced pain concomitantly with attenuation of formalin-evoked glutamate release and enhancement of noradrenaline levels in the spinal DH. GABA levels were not modified. These data suggest that pharmacological blockade of σ_1R reduces peripheral activating glutamatergic nociceptive inputs and enhances noradrenergic descending inhibitory inputs to the DH, but it does not modify the activity of GABAergic inhibitory DH interneurons. Interestingly, i.t. pretreatment with the alpha 2(α_2)-adrenergic receptor antagonist idazoxan prevented the systemic antinociceptive effect of E-52862, suggesting that antinociception elicited by σ_1R blockade depends on the activation of descending inhibitory pathways, which results in enhancement of noradrenaline release into the spinal cord and activation of spinal α_2-adrenoceptors. Noradrenaline could act on presynaptic α_2-adrenoceptors on central projections of formalin-sensitive DRGs to inhibit glutamate release to the superficial DH laminae. Glutamate is released into the DH spinal cord following activation of sensory afferents and its sustained release following sustained stimulation of nociceptors promotes plastic changes leading to spinal amplification of nociceptive messages. Thus, this excitatory amino acid plays a major role in central sensitization phenomena, including wind-up, and the behavioral manifestations of pain sensitization/hypersensitivity (D'Mello and Dickenson 2008; Latremoliere and Woolf 2009). Noradrenaline plays a major role in descending pathways that influence nociceptive signaling in the DH of the spinal cord. Descending inhibition largely involves the release of noradrenaline in the spinal cord from brainstem nuclei such as the locus coeruleus (LC), acting predominantly at the α_2-adrenoceptors, and inhibiting transmitter release from primary afferent terminals and suppressing firing of projection neurons in the DH (Millan 2002; D'Mello and Dickenson 2008). The descending noradrenergic pathways from the brainstem to the DH may also undergo plastic changes in chronic pain states, which results in an increased inhibitory drive that has been suggested to be a homeostatic mechanism counteracting the increased spinal excitability (D'Mello and Dickenson 2008). Accordingly, the finding that E-52862 inhibited formalin-evoked glutamate but enhanced noradrenaline release in the DH (Vidal-Torres et al. 2014) is in agreement with a

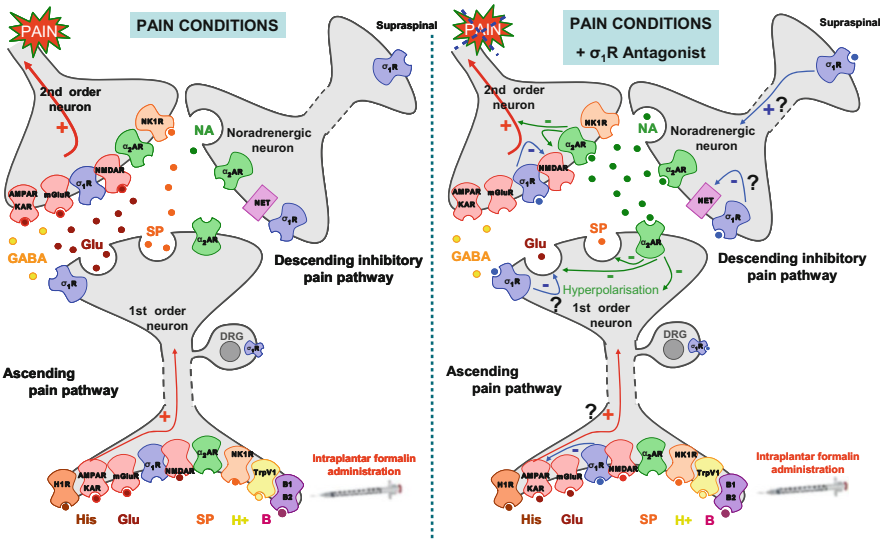

Fig. 2 σ_1R involvement in pain modulation: neurochemistry studies in the spinal cord. Two major pathways are involved in the mechanism of action of σ_1R antagonism in the formalin-induced pain: the inhibition of the spinal excitatory synaptic transmission (Glutamate, Glu levels reduction) and the activation of descending inhibitory systems (Noradrenaline, NA levels enhancement) (Vidal-Torres et al. 2014). Regarding dorsal horn (DH) Glu levels reduction, we hypothesize that σ_1R antagonism reduces the formalin-induced increase in Glu levels by: (1) a direct σ_1R-mediated inhibition of Glu release from the central DRG endings (modulated by σ_1R located presynaptically at the DH central endings or/and postsynaptically at the peripheral endings, which would equally involve hyperpolarization of the first order neuron) or/and (2) an indirect presynaptic, NA-mediated inhibition of Glu release from central afferent endings through presynaptic α_2-adrenoreceptors. This inhibition on Glu release would result in lower activation of NMDAR in postsynaptic second order neurons transmitting pain to upper CNS areas. Regarding DH NA levels increase, σ_1R antagonism-induced enhancement of NA levels could be a consequence of direct σ_1R-mediated: (1) direct increase of NA release at the DH, (2) NA degradation inhibition, (3) inhibition of NA reuptake (NET), or/and (4) activation of supraspinal NAergic neurons projecting to the DH. In any case, increased NA spinal levels are known to produce antinociception via: (1) activation of α_2-adrenoreceptors located presynaptically in primary central afferents, which ultimately results in a reduction of Glu and substance P release from the central endings and (2) postsynaptic activation of α_2-adrenoreceptors located in second order DH neurons, then hyperpolarizing DH neurons and reducing the NMDAR-induced increase of NR1 subunit phosphorylation

modulatory role of σ_1R antagonists in activity-dependent plastic changes, by promoting plasticity of descending inhibitory pathways and stopping down the plastic excitatory synaptic strengthening in the DH (Fig. 2).

9 Pharmacological Vs Genetic Modulation of the σ_1R in Pain: Similarities and Differences

As mentioned in the previous sections, there is plenty of evidence supporting the modulatory role of σ_1R in nociception, mainly based on the pain-attenuated phenotype of σ_1R KO mice and on the antinociceptive effect exerted by σ_1R antagonists. The focus of this section falls on analyzing similarities and differences in the antihypersensitivity profile when using genetic (σ_1R KO) and pharmacological (σ_1R antagonists) approaches. Three different scenarios have emerged (Fig. 3):

- σ_1R-KO mice develop pain similarly to WT mice and σ_1R antagonists exert no antinociceptive effect in WT mice.
- σ_1R-KO mice do not develop pain or pain is attenuated and σ_1R antagonists exert antinociceptive effect in WT mice.
- σ_1R-KO mice develop pain similarly to WT mice and σ_1R antagonists exert antinociceptive effect in WT mice.

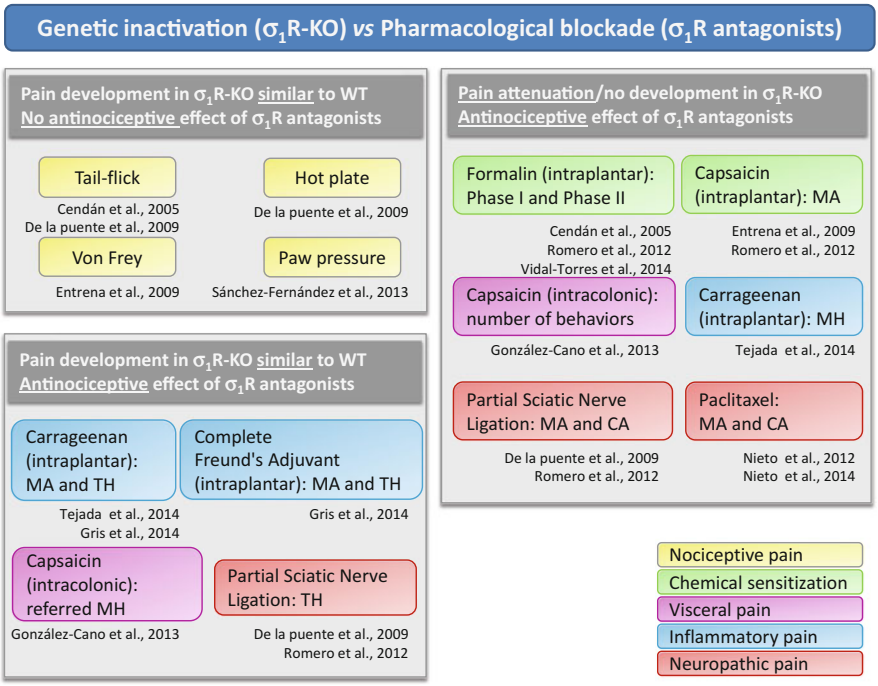

Fig. 3 Genetic inactivation (σ_1R KO) versus pharmacological blockade (σ_1R antagonists). Similarities and differences in their impact in several pain models. *MA* mechanical allodynia, *MH* mechanical hyperalgesia, *TH* thermal hyperalgesia, *CA* cold allodynia, *KO* knockout, *WT* wild type

σ_1R KO mice are a useful genetic tool to study the involvement of σ_1R in several pain types, given that naïve KO mice perceive and respond normally to stimuli of different nature, including mechanical and thermal ones. Thus, the absence of σ_1R in KO mice has been shown to not interfere with the perception of several acute pain stimuli or with the motor response required for paw withdrawal (de la Puente et al. 2009; Entrena et al. 2009b; Nieto et al. 2012; Gonzalez-Cano et al. 2013; Sanchez-Fernandez et al. 2013; Gris et al. 2014; Tejada et al. 2014). In the same way and unlike opioid drugs, σ_1R antagonists fail to modify pain by themselves in classical models of thermal and mechanical acute nociception, as seen in the tail-flick, the hot plate, the von Frey, and the paw pressure tests in rodents (Marrazzo et al. 2006; Entrena et al. 2009a; Sanchez-Fernandez et al. 2013). σ_1R KO mice showed attenuated pain responses in both phases of the formalin test (Cendan et al. 2005b) and did not develop mechanical hypersensitivity following capsaicin sensitization (Entrena et al. 2009b). The pharmacological antagonism of σ_1R produced similar results. Accordingly, the nonselective σ_1R antagonists haloperidol and its metabolites I and II and also E-52862 inhibited formalin-induced pain (Cendan et al. 2005a; Romero et al. 2012) and somatic capsaicin-induced sensitization in mice (Entrena et al. 2009a; Romero et al. 2012).

Regarding neuropathic pain models, cold and mechanical hypersensitivity were strongly attenuated in σ_1R KO mice treated with paclitaxel (Nieto et al. 2012) or exposed to partial sciatic nerve ligation (PSNL) (de la Puente et al. 2009). However, σ_1R KO mice developed thermal hyperalgesia following PSNL (de la Puente et al. 2009). Interestingly, the pharmacological antagonism of σ_1R produced beneficial effects in all of these pain-related manifestations in WT mice. Chronic administration with σ_1R antagonists prevented the development of cold and mechanical allodynia induced by paclitaxel (BD1063 and E-52862) or PSNL (E-52862) in WT mice (Nieto et al. 2012; D'Mello and Dickenson 2008). E-52862 also prevented the development of thermal hyperalgesia induced by PSNL, although this behavior is present in the σ_1R KO mice (D'Mello and Dickenson 2008). Moreover, the acute administration of σ_1R antagonists dose-dependently reversed both paclitaxel- or PSNL-induced hypersensitivity after it had fully developed (D'Mello and Dickenson 2008; Roh et al. 2011). From a mechanistic point of view, σ_1R KO did not show increased diphosphorylated extracellular signal-regulated kinase (pERK) in the spinal cord after paclitaxel administration or PSNL (Roh et al. 2011; de la Puente et al. 2009). Thus, reduced ERK activation could contribute to the observed effects after pharmacological blockade or σ_1R genetic inactivation. In the intracolonic capsaicin visceral pain model, σ_1R KO mice have shown a reduction in the number of pain behaviors as compared to WT mice but developed referred mechanical hyperalgesia similar to WT mice (Chien and Pasternak 1995). Several σ_1R antagonists (i.e., BD1063, NE100, and E-52862) inhibited the number of behavioral responses induced by capsaicin and also reversed the referred mechanical hyperalgesia to the control threshold in WT mice (Chien and Pasternak 1995). These drugs produced no change in σ_1R KO mice, supporting a σ_1R-related mechanism for their effects.

Two different models of inflammatory pain have been explored in σ_1R KO mice, the acute inflammation induced by carrageenan and the chronic inflammation induced

by Complete Freund's Adjuvant (CFA). In the carrageenan-induced inflammatory pain model, σ_1R KO mice did not develop mechanical (paw pressure) hyperalgesia (Tejada et al. 2014) but developed mechanical (von Frey) allodynia and thermal (radiant heat) hyperalgesia (Gris et al. 2014; Tejada et al. 2014). σ_1R antagonists (i.e., BD1063 and E-52862) reversed inflammatory mechanical hyperalgesia, mechanical allodynia, and thermal hyperalgesia in WT mice, an effect which is reduced when combined with the σ_1R agonist PRE-084. However, this effect was mediated by σ_1R as BD1063 and E-52862 had no effect on thermal hyperalgesia and mechanical allodynia in σ_1R KO mice (Gris et al. 2014; Tejada et al. 2014). The antiedematous effects do not account for the decreased hyperalgesia, since carrageenan-induced edema was unaffected in σ_1R KO or by systemic σ_1R pharmacological antagonism (Tejada et al. 2014; Gris et al. 2014). Like in carrageenan-induced inflammatory pain model, the genetic inactivation of σ_1R failed to prevent the development of CFA-induced mechanical allodynia (von Frey filaments). However, the σ_1R antagonist E-52862 reversed CFA-induced mechanical allodynia only in WT mice, but not in KO mice, supporting an on-target mechanism for the effects of this drug (Gris et al. 2014).

Taken together, these data indicated that the lack of σ_1R clearly impacts on the development of neuropathic pain but it did not impact on acute nociceptive pain and partially on the development of inflammatory or visceral pain. Because nociceptive, neuropathic, visceral, and inflammatory pains are known to involve different pathways, the different phenotypes observed in σ_1R KO mice suggest, depending on the pain model and the readout, a different involvement of the σ_1R system in the mechanisms underlying hypersensitivity (Gris et al. 2015). In contrast, systemically administered σ_1R antagonists provided efficacy in all pain-related behaviors evaluated in WT mice (except in acute nociceptive pain), including those developed by σ_1R KO mice (i.e., mechanical allodynia and thermal hyperalgesia induced by carrageenan, mechanical allodynia induced by CFA, referred mechanical hyperalgesia induced by intracolonic capsaicin, and thermal hyperalgesia induced by PSNL) (Fig. 3). This fact brings out the difference between the effect of genetics (i.e., the absence of the receptor and associated adaptive changes) and the pharmacological blockade of σ_1R (i.e., the modulatory effect of a ligand at the time of the test) (Gris et al. 2015; Zamanillo et al. 2013).

Several possible explanations may account for the different analgesic effect profiles generated by genetic and pharmacological approaches. First, some of the differences could be attributed to the lack of selectivity of many of the σ_1R antagonists used in the literature, in contrast to the complete and specific inhibition in σ_1R KO. In fact, many compounds of very different structural classes and with different therapeutic applications, such as antipsychotics (e.g., haloperidol and chlorpromazine), antidepressants (e.g., fluvoxamine, sertraline, and clorgyline), antitussives (carbetapentane, dextromethorphan, and dimemorfan), drugs for the treatment of neurodegenerative disorders such as Parkinson's disease (amantadine) or Alzheimer's disease (memantine and donepezil), and drugs of abuse (cocaine and methamphetamine) can bind, with high to moderate/weak affinity and with no selectivity, to σ_1R and some of them have been used (e.g., haloperidol) to characterize σ_1R pharmacology (Zamanillo et al. 2013; Almansa and Vela 2014). Other compounds (e.g., panamesine, rimcazole, eliprodil,

and others) have been developed as σ_1R ligands, but their selectivity against σ_2R and/or other targets is far from optimal. However, the lack of selectivity cannot be longer supported as an explanation considering results coming from selective compounds such as E-52862. E-52862 shows high affinity for σ_1R (Ki = 17 nM) and a good σ_1R/σ_2R selectivity ratio (>500). Moreover, it is selective over a panel of 170 molecular targets. It behaves as an antagonist, penetrates the blood–brain barrier, and binds to σ_1R in the CNS. Occupancy of σ_1R in the CNS by E-52862 significantly correlated with its antinociceptive effects (Romero et al. 2012). The use of E-52862 as a highly selective σ_1R antagonist has provided a good pharmacological tool to really assess the role of σ_1R in pain modulation. Furthermore, and even more convincing, its activity disappears when administered to σ_1R KO mice.

A second possible explanation is that, unlike the pharmacological treatment in WT mice which produces temporary blockade of σ_1R, σ_1R KO mice are completely deficient in σ_1R function throughout development and adult life. We therefore speculate that pain-related behaviors developed in σ_1R KO mice may be related to the developmental effects of global σ_1R deletion and this cannot be mimicked by treating with antagonists to adult WT mice. Although our initial characterization of σ_1R KO mice did not reveal any overt phenotype, compared with their WT litter mates (Langa et al. 2003), some subtle changes at the level of gene expression may exist throughout life, leading to altered neuroadaptation. This notion of a unique, early development effect by the genetic KO approach has been suggested to account for the discrepancy between genetic KO and pharmacological blockade approaches (Gingrich and Hen 2000). Thus, some effects may not be due to the absence of the receptor in the adult mouse but to the lack of the receptor at some earlier point in development. This has been shown for serotonin 5-HT_{1A} receptors. A developmentally controlled rescue strategy showed that postnatal developmental expression of 5-HT_{1A} receptors is important to establish anxiety-like behavior in adult mice (Gross et al. 2002). In addition, there are studies reporting compensatory effects and conflicting results between pharmacological and genetic inactivation in different cases such as the role of adenosine A_{2A} receptors in psychostimulant-induced behavioral responses and gene expression profiles (Chen et al. 2000, 2003; Yu et al. 2005), the role of 5-HT_7 receptors in depression (Guscott et al. 2005), GABAergic modulation of seizure activity (Voss et al. 2010), endocannabinoid signaling (Min et al. 2010), and the role of δ-subunit-containing γ-aminobutyric acid subtype A receptors in nociception (Bonin et al. 2011), among others. Conditional/inducible mutation approaches, that first allow the mouse to develop and mature normally prior to ablation of the gene of interest, could be of interest to understand discrepancies noted between pharmacological and genetic inactivation.

A third possibility arises from the chaperone nature of σ_1R, which exert their action by physical protein–protein interactions. Accordingly, the absence of the regulatory mechanism in KO mice is not equivalent to the decrease or gain of function promoted by a σ_1R ligand through conformational changes relating to and affecting the activity of the target protein with which σ_1R interacts. In other words, the absence of the modulatory system, as in KO mice, precludes the regulation by ligands, but it does not mimic the modulatory effect elicited by a σ_1R ligand.

10 Concluding Remarks

The effects reported with σ_1R ligands (pronociceptive in the case of agonists and antinociceptive in the case of antagonists) are consistent with a role for σ_1R in central sensitization and pain hypersensitivity and suggest a potential therapeutic use of σ_1R antagonists for the management of neuropathic pain and other pain conditions including inflammatory, visceral, ischemic, postoperative, and orofacial pain. The σ_1R acts as a modulator of the intracellular signaling incurred upon activation of several receptors, enzymes, and ion channels relevant in pain transmission and processing, but the σ_1R is devoid of its own specific signaling machinery. Ligands acting on σ_1R can amplify or reduce the signaling initiated when the target protein that the σ_1R is interacting with becomes activated, but they are per se inactive. On this basis, σ_1R ligands have been postulated as ideal therapeutic drugs, effective only under pathological conditions, but inactive in normal resting/healthy conditions. Thus, while having no effects by themselves, σ_1R ligands exert their modulatory activity under conditions involving a disturbance, such as chronic pain. In other words, under normal physiological conditions most target proteins are not affected by σ_1R ligands. This concept is very important in terms of safety and tolerability, as an ideal analgesic drug should be able to modify the stressed/dysfunctional pathway without affecting normal physiological functions. In the case of σ_1R antagonists, no adverse events have been described in rodents at doses exerting antinociceptive effects based on preclinical studies. Unlike other analgesics (e.g., opioids), σ_1R antagonists do not modify the normal sensory perception, and normal/baseline nociceptive thresholds are not modified when σ_1R antagonists are administered to normal animals. Only when the system is sensitized and hypersensitivity (i.e., allodynia and hyperalgesia) occurs following prolonged noxious stimulation (e.g., capsaicin or formalin injection) or persistent abnormal afferent input (e.g., nerve injury or inflammation), a σ_1R antagonist can exert its effect, which is the reversion of the diminished pain thresholds back to normal sensitivity thresholds. Accordingly, σ_1R antagonists are not strictly analgesics; they are antiallodynic and antihyperalgesic drugs. Moreover, there is plenty of data supporting the combination of σ_1R antagonists with opioid therapy, which may result in a potentiation of opioid analgesia without significant increase in unwanted effects. These observations mean that lower doses of opioids, with less side effects but efficacious based on the selective enhancement of the analgesic effect, could be potentially used if σ_1R antagonists are used as opioid adjuvants.

Overall, based on the preclinical data, the use of selective σ_1R antagonists could represent a promising efficacious and safe strategy to approach difficult-to-treat chronic pain conditions including neuropathic pain, and to enhance analgesic efficacy and increase the safety margin of opioids. In this regard, the most advanced investigational σ_1R antagonist, E-52862, exhibited an acceptable safety, tolerability, pharmacodynamic, and pharmacokinetic profile in phase I studies and is now in phase II studies in chronic neuropathic pain and in postoperative pain in combination with morphine. The outcome of clinical studies with the σ_1R antagonist E-52862 will be of great interest to assess the potential of this new therapeutic approach to pain management.

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Allosteric Modulation of Opioid G-Protein Coupled Receptors by Sigma$_1$ Receptors

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Contents

Abstract

Since their proposal in 1976, the concept of sigma$_1$ receptors has been continually evolving. Initially thought to be a member of the opioid receptor family, molecular studies have now identified its genes and established its structure crystallographically. Much effort has now revealed its importance as a chaperone in the endoplasmic reticulum, but its functions extend beyond this. Sigma$_1$ receptors have been associated with a host of signaling systems. Evidence over the past 20 years has established the modulatory effects of sigma$_1$ ligands on opioid systems. Despite their inability to bind directly to opioid receptors, sigma$_1$ ligands can modulate

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opioid analgesia in vivo and signal transduction mechanisms in vitro. Furthermore, sigma$_1$ receptors can physically associate with GPCRs. Together, these findings show that sigma$_1$ ligands can function as allosteric modulators of GPCR function through their association with the sigma$_1$ receptors, which are in direct physical association with opioid receptors, members of the G-protein coupled family of receptors.

Keywords
Allosteric modulator • Allosterism • Analgesia • GPCR • Opioid • Sigma$_1$ receptor • Transduction

1 Introduction

Sigma receptors were first proposed by Martin based upon the pharmacology of the benzomorphan SKF10,047 (Martin et al. 1976). In this initial report, Martin found that the actions of SKF10,047, including the behavioral effects, were reversed by the classical opioid antagonist naltrexone, leading him to consider this an opioid receptor. Since then, the concept of sigma$_1$ receptors has evolved. Since the (−)benzomorphans SKF10,047 and (−)pentazocine also label traditional opioid receptors with high affinity, their (+)isomers are now used to define sigma$_1$ receptors, clearly separating them from opioid receptors that are highly selective for (−)isomers. Today the term refers to a protein with a structure unrelated to any established class of receptor (Hanner et al. 1996; Kekuda et al. 1996; Seth et al. 1997, 1998; Pan et al. 1998; Mei and Pasternak 2001). The crystal structure of the sigma$_1$ receptor has been published (Schmidt et al. 2016). It has a trimeric architecture with a single transmembrane domain and an intracellular binding pocket for each monomer. The function of sigma$_1$ receptors has remained enigmatic. Unlike classical receptors, it has no identified transduction systems. It has been associated with the endoplasmic reticulum and has chaperone actions (Hayashi and Su 2007) and associates with many proteins in various classes, many of them receptors and channels (Monnet et al. 1996; Aydar et al. 2002; Su and Hayashi 2003; Martina et al. 2007; Kim et al. 2010).

This chapter will focus on the interactions of sigma$_1$ receptors with opioid receptors, members of the G-protein coupled receptor (GPCR) family. It will review the evidence for a modulatory role of sigma$_1$ receptors on GPCR function involving select actions and locations within the central nervous system, extending the general actions of sigma$_1$ receptors beyond their chaperone actions.

2 Sigma$_1$ Modulation of Opioid Actions in Mice

2.1 Modulation of Morphine Analgesia by (+)Pentazocine and Haloperidol in Mice

Opiate analgesics are often used in combination with other classes of drugs in the hope of enhancing analgesia while limiting side effects (Payne and Pasternak 1992, 1998). These adjuncts cover a wide range of classes, from antihistamines to antidepressants. While some are analgesic, others are not. In the course of exploring the pharmacology of the stereoisomers of pentazocine, we observed interesting interactions between (+)pentazocine and opioid actions (Chien and Pasternak 1993, 1994, 1995a, 1995b; Pasternak 1994; Chien et al. 1997; King et al. 1997; Pan et al. 1998; Mei and Pasternak 2001, 2002, 2007; Pasternak 2007).

(−)Pentazocine is a mixed kappa agonist/mu antagonist with high binding affinity at both mu and kappa opioid receptors. Clinically, in the USA, it is supplied as a racemate, containing both the (−) and (+)isomers, leading us to look at each independently. In view of its antagonist properties against mu opioid receptors, it was not surprising to observe that the pentazocine lowered morphine analgesia in a mouse model. However, when the two stereoisomers were examined, they were equipotent (Fig. 1a) (Chien and Pasternak 1994). Since (+)pentazocine does not have appreciable affinity for any of the opioid receptors, it was assumed that the lowering of morphine analgesia was secondary to interactions with sigma receptors. This was confirmed in several ways. Haloperidol is an established neuroleptic that is active against both sigma$_1$ asnd D2 dopamine receptors. Haloperidol potentiated morphine analgesia, shifting its analgesic ED_{50} twofold in CD-1 mice (Fig. 1b). This effect was independent of its actions on D2 dopamine receptors. (−)Sulpiride is a potent neuroleptic active at D2 dopamine receptors that differs from haloperidol in that (−)sulpiride does not bind to sigma$_1$ receptors. Unlike haloperidol, (−)sulpiride did not potentiate morphine analgesia. In addition, haloperidol, but not sulpiride, reversed the diminished morphine response due to (+)pentazocine (Fig.1b). Studies in a D2 dopamine receptor knockout mouse showed that dopamine receptors can influence opioid analgesia directly, but haloperidol still enhanced morphine analgesia in the D2 knockout mice, validating the role of sigma$_1$ receptors in the modulation of opioid analgesia (King et al. 2001).

2.2 Role of Sigma$_1$ Receptors in Delta and Kappa Opioid Analgesia and Strain Differences in Mice

Like morphine analgesia, the sigma$_1$ system impacts both delta and kappa opioid analgesia (Fig. 1c; Table 1). (+)Pentazocine decreased, in a dose-dependent manner, the analgesic actions of the delta opioid peptide DPDPE ([D-Ala^2,D-Pen^2,D-Pen^5]enkephalin), the kappa$_1$ opioid U50,488H, and naloxone benzoylhydrazone (NalBzoH), which acts through a truncated splice variant of the mu opioid receptor gene *Oprm1* (Pan et al. 2001; Majumdar et al. 2011; Lu et al. 2015). In vivo, sigma$_1$ receptors are tonically active, initially indicated by the ability of haloperidol to enhance

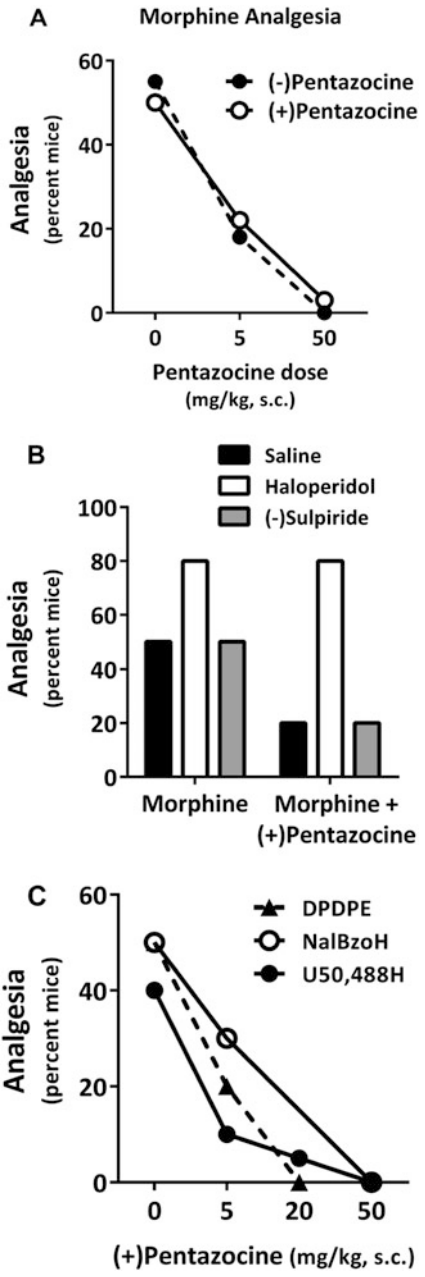

Fig. 1 Effect of (+)pentazocine on opioid analgesia in mice. (**a**) Groups of mice ($n = 20$) received morphine (5 mg/kg, s.c.) alone or with the indicated dose of the specified pentazocine stereoisomer. (**b**) Groups of mice ($n = 20$) received morphine (5 mg/kg, s.c.) alone or with haloperidol (0.5 mg/kg, sc.) or (−)sulpiride (200 mg/kg, s.c.) (*left side*). Additional groups received the same treatments along with (+)pentazocine (5 mg/kg, s.c.). (**c**) Groups of mice ($n = 10$) received U50,488H (5 mg/kg, s.c.), naloxone benzoylhydrazone (NalBzoH) (50 mg/kg, s.c.), or [D-Ala^2,D-Pen^2,D-Pen^5] enkephalin (DPDPE) (300 ng, i.t.) alone or with the indicated dose of (+)pentazocine. Data from the literature (Chien and Pasternak 1994)

morphine analgesia. Similarly, haloperidol significantly potentiated the actions of U50,488H and NalBzoH. The inactivity of (−)sulpiride confirmed a role for sigma$_1$,

Table 1 Effect of haloperidol on opioid analgesic potency

Opioid	Strain	ED_{50}				Ratio
		Control		Haloperidol		
Morphine	CD-1	4.5	mg/kg, s.c.	2.3	mg/kg, s.c.	2
DPDPE	CD-1	312	ng, i.t.	103	ng, i.t.	3
U50,488H	CD-1	4.8	mg/kg, s.c.	1.8	mg/kg, s.c.	2.7
	BALB-C	16.0	mg/kg, s.c.	1.9	mg/kg, s.c.	8.9
NalBzoH	CD-1	55.3	mg/kg, s.c.	21.4	mg/kg, s.c.	2.6
	BALB-C	10% response at 100 mg/kg		23.2	mg/kg, s.c.	

ED_{50} values were determined for the indicated drug/route in the specified mouse strain. The ratio of ED_{50} values is also presented. In addition to enhancing analgesic potency with shifts to the left of the ED_{50} values, haloperidol also eliminated the strain differences in potency for U50,488H and NalBzoH seen in control values. Data from the literature (Chien and Pasternak 1994)

and not D2 dopamine receptors. Thus, sigma$_1$ receptors modulate the analgesic activity of all three classes of opioid receptors: Mu, Delta, and Kappa.

Patients vary markedly in their sensitivity to opioids, with some requiring far higher doses than others (Payne and Pasternak 1992, 1998). These differences are recapitulated in various mouse strains, which also show marked differences in their sensitivity to opioids (Table 1) (Moskowitz et al. 1985; Pick et al. 1991; Marek et al. 1993; Mogil et al. 1995). This was quite pronounced with the kappa drugs U50,488H and NalBzoH. U50,488H was almost fourfold more potent in CD1 than in BALB-C mice. A greater difference was observed for NalBzoH, where a dose almost twice the ED_{50} in CD-1 mice gave less than 10% response in BALB-C mice. In both cases, haloperidol increased their potency. However, haloperidol also eliminated the differences in potency between the two strains (Table 1) (Chien and Pasternak 1994). Thus, tonic activity of sigma$_1$ receptors may contribute to strain differences in analgesic sensitivity.

2.3 Non-analgesic Actions

Opioids such as morphine have a range of actions other than analgesia, including inhibition of gastrointestinal (GI) transit, which contributes to their constipating effect. Although both morphine analgesia and the inhibition of GI transit are mediated through mu receptors, they differ in their sensitivity to several selected antagonists, including naloxonazine (Heyman et al. 1988; Paul and Pasternak 1988). These observations provided some of the first suggestions that it may be possible to separate analgesia from undesired side effects. Differences between morphine analgesia and GI transit extend to their sensitivity to sigma$_1$ systems as well.

Morphine diminishes GI transit in a dose-dependent manner. Although (+)pentazocine effectively lowered morphine analgesia, it had no effect upon morphine-induced inhibition of GI transit (Fig. 2) (Chien and Pasternak 1994). The inhibition of transit was equivalent with and without (+)pentazocine at both morphine doses.

Morphine lethality results, in large part, by its respiratory depressant effects. At a morphine dose that was lethal in 70% of mice, (+)pentazocine had no effect, implying

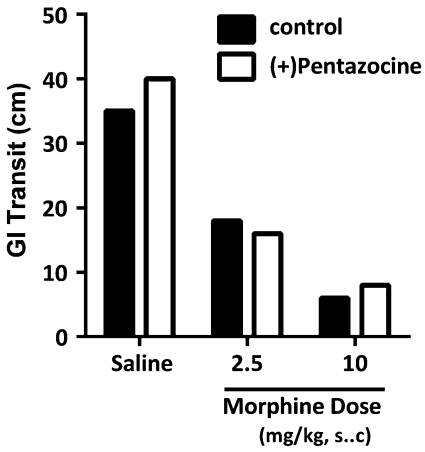

Fig. 2 Effect of (+)pentazocine on morphine-induced inhibition of gastrointestinal transit. Groups of mice received either saline or the stated doses of morphine alone or with (+)-pentazocine at a dose (5 mg/kg, s.c.) that reduces systemic morphine analgesia by approximately 50%. Data from the literature (Chien and Pasternak 1994)

that sigma$_1$ receptors do not modulate this activity (Chien and Pasternak 1994). This selective increase of analgesia and not either lethality or inhibition of GI transit may prove useful clinically by increasing the therapeutic index.

3 Sigma$_1$ Modulation of Opioid Actions in Rats

The actions of σ drugs on opiates were not limited to mice. Rats displayed similar effects (Chien and Pasternak 1995b; Mei and Pasternak 2007). (+)Pentazocine reduced the analgesic actions of all classes of opioids. Like mice, rats also displayed tonic sigma$_1$ activity, as evidenced by the ability of haloperidol to potentiate analgesia. Although haloperidol was inactive alone in the tail flick assay, it enhanced morphine, U50,488H, and NalBzoH analgesia in rats. Again, (−)sulpiride was ineffective, confirming a sigma$_1$ mechanism. However, these interactions became far more complicated when examined in specific regions of the brain, specifically the periaqueductal gray (PAG), the rostral ventral medulla (RVM), and the locus ceruleus (LC) (Mei and Pasternak 2007).

Morphine is a potent analgesic when administered directly into various regions of the brain (Pert and Yaksh 1974; Rossi et al. 1994; Mei and Pasternak 2007). (+)Pentazocine diminishes morphine analgesia when both drugs are given systemically, but a different pattern emerged when specific brainstem loci were examined (Mei and Pasternak 2007). (+)Pentazocine potently reduced morphine analgesia in a dose-dependent manner in both the RVM and the LC at doses under 1 μg, but not in the PAG (Fig. 3a). These microinjection studies also revealed another difference. While haloperidol significantly enhanced the response in the RVM, it was not effective in either the PAG or LC (Fig. 3b). The lack of a haloperidol effect in the PAG is consistent with its insensitivity to (+)pentazocine which suggests the absence of a sigma$_1$ contribution to mu analgesia in this site. However, sigma$_1$ receptor is

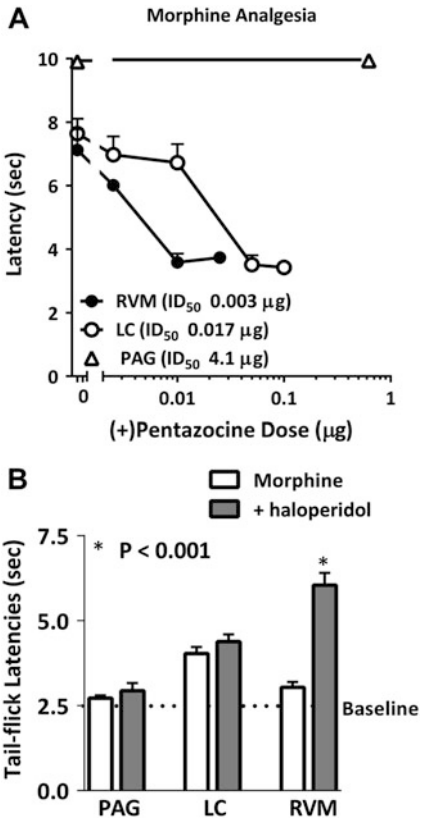

Fig. 3 Effect of microinjection of (+)pentazocine in rat brainstem regions. (**a**) Dose–response relationship of (+)-pentazocine on morphine analgesia was assessed at peak effect in each of the regions noted. Analgesia is shown as %MPE to facilitate comparisons among regions. ID_{50} values with 95% confidence limits were 4,090 ng (830, 5,470) in the periaqueductal gray (PAG), 17.4 ng (9.1, 25) in the LC, and 2.6 ng (0.1, 5.3) in the rostral ventral medulla (RVM). (**b**) Groups of rats ($n = 6$) with cannulae in either the PAG, RVM, or LC received morphine 10 min after either saline or haloperidol (5 µg), and a time–action curve for analgesia was obtained. Peak effects were determined for each group

modulatory in the LC, so the lack of a haloperidol effect there more likely implies the absence of tonic sigma$_1$ activity.

Together, these observations indicate that sigma$_1$ receptor modulation of morphine action is not universal. The rat microinjection studies show that its actions are restricted to selected regions of the brain. Furthermore, sigma$_1$ receptors are not involved with either the inhibition of GI transit or lethality. Thus, the modulation of opioid actions by sigma$_1$ receptors is itself modulated.

4 Molecular Studies of Sigma$_1$ Receptors

For many years, a number of questions were raised regarding the sigma$_1$ receptor, including its structure. The cloning of the sigma$_1$ receptor was a major advance in our understanding of the protein (Hanner et al. 1996; Kekuda et al. 1996; Seth et al. 1997, 1998; Pan et al. 1998; Mei and Pasternak 2001). Despite its close association with opioid receptors, the sigma$_1$ receptor shows no homology to traditional GPCRs, such as the opioid receptors. It is relatively small and the recent crystallization of the protein reveals a trimeric structure with each monomer containing a single transmembrane

domain with a large intracellular C-terminus that contains the binding pocket (Schmidt et al. 2016). When expressed, the cloned sigma$_1$ receptor retained all the binding characteristics expected from earlier studies from brain and other tissues. This validation was important since many of the drugs used to characterize the protein were not selective. More detailed analysis has now identified a number of alternatively spliced variants of the sigma$_1$ receptor (Pan et al. 2017), but their significance has not yet been fully defined.

A persistent question is whether or not the protein is actually a receptor. The ability to demonstrate opposing actions with different chemical structures led to the widespread use of the terms agonist and antagonist, despite the absence of a defining function for the protein. Conceptually, the designation of antagonist is supported by antisense studies (King et al. 1997; Pan et al. 1998). Downregulation of sigma$_1$ receptors using antisense oligodeoxynucleotides administered centrally potentiated opioid analgesia, actions similar to those of "antagonists." Downregulation of sigma$_1$ receptor mRNA by approximately 65% shifted the analgesic activity of morphine, U50,488H, DPDPE, and NalBzoH by three- to fourfold (Table 2) (Pan et al. 1998). Thus, the actions of "antagonists" corresponded to the loss of the protein. As our understanding of the protein at the molecular level has advanced, no transduction system associated with the protein has been identified. However, the long history of the use of the terms "receptor," "antagonist," and "agonist" is heavily entrenched and not likely to be abandoned.

4.1 Allosteric Modulation of Opioid Receptor Transduction by Sigma$_1$ Receptors

The behavioral studies implied an allosteric modulation of opioid receptors by sigma$_1$ receptors. This concept is supported by molecular studies exploring the interactions of mu opioid receptors and sigma$_1$ receptors (Kim et al. 2010). Sigma$_1$ antagonist BD1047 did not compete opioid receptor binding, confirming that it has no direct interaction with the opioid receptors, and did not stimulate ^{35}S-GTPγS binding by

Table 2 Effect of antisense targeting the sigma$_1$ receptor on opioid analgesia

Drug	ED_{50}						Antisense shift
	Saline		Mismatch		Antisense		
Morphine	5.3	mg/kg, s.c.	5.5	mg/kg, s.c.	1.9	mg/kg, s.c.	3
U50,488H	10	mg/kg, s.c.	9.7	mg/kg, s.c.	3.4	mg/kg, s.c.	3
DPDPE	6.1	i.c.v.	6.4	i.c.v.	2.1	μg, i.c.v.	3
NalBzoH	96	mg/kg, s.c.	91	mg/kg, s.c.	19.7	mg/kg, s.c.	4.6

ED_{50} values were determined for each drug in CD-1 mice following treatment with either saline, an inactive mismatch oligodeoxynucleotide, or an antisense oligodeoxynucleotide. Data from the literature (Pan et al. 1998)

itself. Inclusion of BD1047 also did not change the Bmax of ^{3}H-DAMGO binding, a measure of the number of the receptors in the assay. However, BD1047 significantly potentiated the ability of the mu opioid peptide DAMGO to stimulate ^{35}S-GTPγS binding in brain, shifting the ED_{50} more than 25-fold to the left (Fig. 4). This stimulation was limited to a shift in the potency, with no change in the maximal stimulation. This raises an interesting issue. Since the affinity of the opioid was unaffected, the shift in the dose–response curve implied a greater intrinsic activity since a similar level of stimulation could now be elicited with a lower receptor occupancy. Thus, BD1047 allosterically modulated the functional actions of DAMGO through its binding to the sigma$_1$ receptor, enhancing its intrinsic activity. Similar effects were observed in cell lines and with other receptor classes, suggesting a more general modulation of GPCR (Kim et al. 2010).

4.2 Physical Association of Sigma$_1$ and Opioid Receptors

The modulation of opioid-induced ^{35}S-GTPγS binding by sigma$_1$ antagonists implied a physical interaction between the two proteins. One advantage of having the cloned receptors is the ability to tag them with different epitopes, enabling their efficient immunoprecipitation. In this approach, the receptor complex is gently solubilized to prevent dissociation of proteins from the receptor complex. The complex is then immunoprecipitated with an antibody to one protein and the material is then examined by Western blot using an antibody against the second protein. When examined, we observed a direct interaction between the sigma$_1$ receptor and the mu opioid

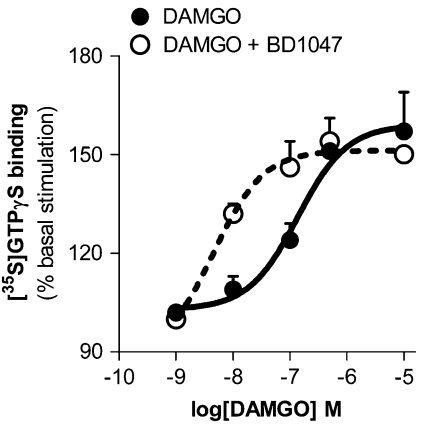

Fig. 4 Effect of the sigma$_1$ antagonist BD1047 on DAMGO stimulation of ^{35}S-GTPγS binding. Dose–response curves of DAMGO-induced stimulation of ^{35}S-GTPγS binding alone (F, *solid line*) or combined with 10 nM BD1047 (E, *broken line*). The EC_{50} of DPDPE alone is 209 and 12.3 nM in the presence of BD1047 (10 nM; $P < 0.002$, determined by two-way ANOVA). *B*max values, 189 and 176%, respectively, are not significantly different. Data from the literature (Kim et al. 2010)

receptor (Fig. 5) (Kim et al. 2010). Only membranes from cell containing both proteins showed evidence of physical association. Also, note that the co-immunoprecipitation also showed the lower molecular weight immature form of Flag-MOR-1, consistent with an association of the two receptors in the endoplasmic reticulum.

5 Conclusion

For many years, sigma$_1$ receptors have raised questions. Their cloning answered many of these and the recent crystallographic structure has given us major insights. However, many questions remain regarding their function. Evidence supports a major role as a chaperone in the endoplasmic reticulum, but their ability to modulate GPCR function suggests a broader range of actions as well. Sigma$_1$ ligands presumably modulate opioid actions by binding to the sigma$_1$ receptor which then modulates opioid function allosterically through protein–protein interactions. This allosteric modulation is not restricted to mu opioid receptors but extends to a number of different GPCRs. However, it is not a universal interaction, as shown by the variations in response in different brain regions, which is consistent with its distribution in the brain, which is not uniform (McCann et al. 1994). In addition, it varies among different pharmacological functions.

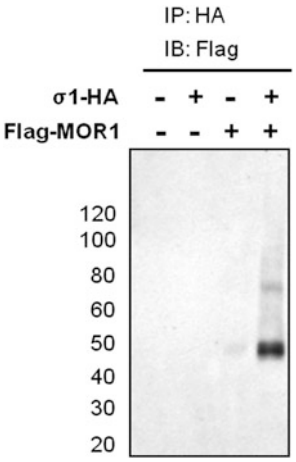

Fig. 5 Co-immunoprecipitation of epitope tagged mu opioid receptor MOR-1 and sigma$_1$ receptors. Co-immunoprecipitation experiments were performed using detergent-soluble cell membranes prepared from HEK293T Flag-MOR-1 and sigma$_1$-HA stably transfected cells. Immunoprecipitated samples were resolved by SDS-PAGE. All apparent molecular masses are represented in kilodaltons. *Left panel*: HA-Sigma$_1$ receptor was immunoprecipitated (IP) with agarose bead-coupled HA antibody and eluted and run on 10% SDS-polyacrylamide gel electrophoresis. To detect Flag-MOR-1, the gel was immunoblotted (IB) with an anti-Flag antibody and a band seen only in lysates from cells coexpressing both Flag-MOR-1 and MOR-1-HA. The mature Flag-MOR-1 is seen at approximately 70 kDa and the immature form (i.e., non-glycosylated) at 45 kDa. Data from the literature (Kim et al. 2010)

Studies of sigma$_1$ receptors are reminiscent of the analogy of the blind men examining an elephant, with each grasping different places on the animal and coming away with very different descriptions of the animal. Sigma$_1$ receptors have a wide range of functions and they all should be explored. They function as chaperones, associate with various cellular systems (Monnet et al. 1996; Aydar et al. 2002; Hayashi and Su 2003, 2007; Martina et al. 2007; Kim et al. 2010), and are involved with a range of diseases, ranging from addiction to pain to psychiatric disorders (Maurice and Su 2009; Crottes et al. 2013; Rousseaux and Greene 2015). Integrating all its activities into a general understanding of sigma$_1$ function should be the ultimate goal of the field.

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A Role for Sigma Receptors in Stimulant Self-Administration and Addiction

Jonathan L. Katz, Takato Hiranita, Weimin C. Hong, Martin O. Job, and Christopher R. McCurdy

Contents

Abstract

Sigma receptors (σRs) are structurally unique proteins that function intracellularly as chaperones. Historically, σRs have been implicated as modulators of psychomotor stimulant effects and have at times been proposed as potential avenues for modifying stimulant abuse. However, the influence of ligands for σRs on the effects of stimulants, such as cocaine or methamphetamine, in various preclinical procedures related to drug abuse has been varied. The present paper reviews the effects of σR agonists and antagonists in three particularly relevant procedures: stimulant discrimination, place conditioning, and self-administration. The literature to date suggests limited σR involvement in the discriminative-stimulus effects of psychomotor stimulants, either with σR

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agonists substituting for the stimulant or with σR antagonists blocking stimulant effects. In contrast, studies of place conditioning suggest that administration of σR antagonists or down-regulation of σR protein can block the place conditioning induced by stimulants. Despite place conditioning results, selective σR antagonists are inactive in blocking the self-administration of stimulants. However, compounds binding to the dopamine transporter and blocking σRs can selectively decrease stimulant self-administration. Further, after self-administration of stimulants, σR agonists are self-administered, an effect not seen in subjects without that specific history. These findings suggest that stimulants induce unique changes in σR activity, and once established, the changes induced create redundant, and dopamine independent reinforcement pathways. Concomitant targeting of both dopaminergic pathways and σR proteins produces a selective antagonism of those pathways, suggesting new avenues for combination chemotherapies to specifically combat stimulant abuse.

Keywords
Chaperone protein • Cocaine • Drug abuse • Methamphetamine • Reinforcing effects • Self-administration • Sigma receptor (σR)

1 Introduction

Due to its initial association with opioid receptors (Martin et al. 1976), a focus of much of the behavioral research on compounds thought to act on σRs has been related to drug abuse. Some of the first compelling findings that σRs may be involved in psychomotor stimulant effects came from studies with the σR antagonists,[1] rimcazole and BMY 14802, that demonstrated blockade of cocaine-induced locomotor stimulation (e.g., Menkel et al. 1991), as well as σR ligand modulation of dopaminergic function (see review by Werling et al. 2007). Further studies showed that locomotor responses enhanced by repeated cocaine or methamphetamine administration were also blocked by σR antagonists (Ujike et al. 1992a, b; Witkin et al. 1993). Still other studies found that convulsive effects and lethality induced by cocaine are blocked by σR antagonists (e.g., Mésangeau et al. 2008), and that σ_1R antisense injected via indwelling cannulas to the lateral

[1]The designation of σR ligands as agonists or antagonists stems from initial conceptions of these proteins as G-protein coupled receptors. As the understanding of these proteins has evolved and it has become clear that they function as intracellular chaperones, there remain observations that ligands for these proteins have different effects and some can block the actions of others (Hayashi and Su 2007; Katz et al. 2016). Most of the designations of these ligands as agonists or antagonists are less than definitive and accrue from in vivo observations that might not meet the strictest pharmacological standards. Absent more definitive results we will designate compounds as agonists or antagonists according to common usage within the scientific community. Complicating the picture are studies indicating subtypes of σRs. Where necessary in discussions herein we use designations of σR subtype selectivity based on radioligand binding results which are shown in Table 1.

Table 1 Affinities of various compounds in specifically binding to σ_1, or σ_2 receptors, as well as subtype selectivity

Compound	σ_1 K_i value (95% CLs)	σ_2 K_i value[a] (95% CLs)	σ_1 K_i value/ σ_2 K_i value	Original report
AC 927	53.1 (45.6–61.8)	78.9 (48.2–129)	0.673	Hiranita et al. (2011a)
Allopregnanolone	3,090 (2,140–4,450)	6,390 (4,320–9,450)	0.484	Previously unpublished
AZ-66	4.70 (4.06–5.45)	1.35 (0.911–2.00)	3.48	Katz et al. (2016)
BD1008	2.13 (1.77–2.56)	16.6 (13.0–21.1)	0.128	Garcés-Ramírez et al.(2011)
BD1047	3.13 (2.68–3.65)	47.5 (36.7–61.4)	0.066	Garcés-Ramírez et al. (2011)
BD1063	8.81 (7.15–10.9)	625 (447–877)	0.014	Garcés-Ramírez et al. (2011)
CM 156	2.02 (1.80–2.25)	0.677 (0.459–1.00)	2.98	Previously unpublished
CM 304	0.684 (0.552–0.847)	388 (215–702)	0.002	Katz et al. (2016)
CM 353	1,120 (905–1,380)	4.48 (2.66–7.55)	250	Katz et al. (2016)
CM 398	1,490 (1,200–1,860)	4.50 (2.78–7.27)	331	Katz et al. (2016)
DTG	57.4 (49.3–66.7)	21.9[b] (14.8–32.4)	2.62	Garcés-Ramírez et al. (2011)
DUP 734	1.25 (1.13–1.38)	45.6 (38.4–54.2)	0.027	Previously unpublished
Fluvoxamine	26.1 (22.7–29.9)	244 (51.1–1,170)	0.107	Previously unpublished
Haloperidol	2.91 (2.69–3.14)	19.6 (15.6–24.6)	0.148	Northcutt et al. (2015)
NE-100	2.48 (2.13–2.88)	121 (91.9–159)	0.020	Hiranita et al. (2011a)
(+)-Pentazocine	4.59[b] (4.26–4.97)	224 (195–257)	0.020	Hiranita et al. (2013b)
PRE-084	53.2 (44.8–63.2)	32,100 (23,100–44,700)	0.002	Garcés-Ramírez et al. (2011)
Progesterone	638 (547–744)	2,760 (898–8,460)	0.231	Previously unpublished
Rimcazole	883 (661–1,180)	238 (171–329)	3.71	Hiranita et al. (2011a)
SH 3-24	22.9 (18.5–28.2)	20.0 (15.7–25.6)	1.15	Hiranita et al. (2011a)
SH 3-28	19.0 (15.3–23.6)	47.2 (40.4–55.2)	0.403	Hiranita et al. (2011a)
(+)-SKF 10,047	58.7 (49.0–70.4)	1,470 (236–9,410)	0.040	Previously unpublished

(continued)

Table 1 (continued)

Compound	σ_1 K_i value (95% CLs)	σ_2 K_i value[a] (95% CLs)	σ_1 K_i value/ σ_2 K_i value	Original report
(±)-SM-21	2,760 (1,700–4,470)	263 (166–409)	10.5	Katz et al. (2016)
SN 79	78.6 (65.2–94.7)	11.3 (7.91–16.3)	6.96	Katz et al. (2016)
SN 167	392 (317–485)	16.1 (10.4–24.9)	24.3	Katz et al. (2016)

The values listed are K_i values with 95% confidence limits (CLs) in parentheses, with exceptions as noted

[a]The values reported for all compounds were determined using identical assay conditions. For the [^{3}H]DTG assay [in the presence of (+)-pentazocine], the data often modeled better for two than one binding site, and the K_i values for the higher affinity site are displayed in the table, as that site is the site recognized as the σ_2R. The low affinity site is currently not identified. Obtained values for the low affinity DTG site and their 95% CLs in nM were as follows: BD1008: 20,500 (9,640–43,500); BD1047: 55,300 (25,000–122,000); BD1063: 53,700 (16,500–174,000); DTG 3,520 (257–4,820); rimcazole: 25,900 (3,620–185,000); SH 3-24:12,700 (1,300–124,000); AC927: 55,200 (6,860–444,000); CM 304: 12,800 (87.7–1,860,000); (±)-SM 21: 30,800 (4,730–200,000): SN 79: 9,130 (2,860–29,100); SN 167: 373,000 (89,500–1,560,000); CM 398: 180,000 (2,230–14,500,000)

[b]The value for affinity of (+)-pentazocine at σ_1, and DTG at σ_2 receptors are K_d values obtained from homologous competition studies

ventricles attenuated the convulsive and locomotor stimulant effects of cocaine, whereas a mismatch sequence was relatively less active (Liu et al. 2007b).

Although the blunting of stimulant-induced locomotor stimulation or acute toxicity has been well documented (see review by Matsumoto et al. 2014), interactions with other behavioral effects closely related to stimulant abuse liability have been explored less thoroughly. The present chapter will review the literature on effects of compounds acting at σRs with regard to various behavioral effects related to the abuse liability of stimulants or the antagonism of effects of stimulants. Close attention will be paid to three behavioral effects: discriminative-stimulus effects of drugs, place conditioning, and drug self-administration. Each of these effects is more closely related to the abuse of drugs per se, than are locomotor stimulation, either sensitized or acute, and the toxicity found at high doses of stimulants. Both σR agonist and antagonist effects will be reviewed as appropriate, and with some exceptions, the focus will be on compounds presently thought to be selective for σRs (Table 1).

2 Drug Discrimination

In these procedures, subjects are placed in a chamber typically with two levers on which the subjects are trained to press with food reinforcement. At the completion of training, responses on only one of the levers intermittently produces reinforcement during sessions preceded by drug injection and responses on the other produce

reinforcement only after vehicle injections. These training sessions are conducted to establish the stimuli arising from drug injection as discriminative for allocation of responses to one of the two levers.[2] Once trained, the subjects can be tested with various doses of the training drug, or doses of other compounds with the degree of responding on the drug-appropriate lever thought to be an indication of shared interoceptive effects and mechanism with the training drug. Additionally, putative antagonists can be tested to determine whether they shift responding on the drug-appropriate lever to the saline lever. A shift down or rightward of the training drug dose-effect curve by a putative antagonist is evidence of physiologic or competitive antagonism by the test compound. Although drug-discrimination studies do not bear directly on the abuse liability of the involved drugs, it has substantial pharmacological specificity, and can be used effectively for various purposes, not the least of which is pharmacological characterization of unknowns (e.g., Holtzman 1985; Schuster and Johanson 1988).

Several studies have assessed the ability of σR ligands to substitute for the discriminative-stimulus effects of psychomotor stimulants (Table 2). For example, Cohen and Sanger (1994) reported a lack of cocaine-like discriminative-stimulus effects of σR ligands (DTG, haloperidol, rimcazole and BMY 14802) in rats, with later replications of those results with DTG and rimcazole (Katz et al. 2003; Ukai et al. 1997). Several other σR ligands (AC 927, SA4503, trishomocubanes) have failed to produce full cocaine-like discriminative-stimulus effects in rats (Liu et al. 2007a; Matsumoto et al. 2011; Rodvelt et al. 2011a). The lack of cocaine-like discriminative-stimulus effects of DTG was extended to the selective σ_1R agonist PRE-084 with various routes of administration (Hiranita et al. 2011b). Moreover, the lack of stimulant-like discriminative-stimulus effects of σR agonists, (+)-pentazocine and SA4503, was substantiated when substituted in rats trained to discriminate methamphetamine or 3,4-methylenedioxymethamphetamine (MDMA) (Mori et al. 2014b; Rodvelt et al. 2011b). Results with σR antagonist pretreatments further support a lack of involvement of σR systems in the discriminative-stimulus effects of stimulants. For example, several σR antagonists (rimcazole, AC 927, NE 100) failed to block the discriminative-stimulus effects of cocaine (Cohen and Sanger 1994; Katz et al. 2003; Matsumoto et al. 2011) or MDMA (Mori et al. 2014b) in rats (Table 2).

The substitution for non-competitive *N*-methyl-D-aspartate (NMDA) antagonists, including phencyclidine (PCP), ketamine, or dextromethorphan by σR agonists has also been examined. In two studies (+)-pentazocine has consistently lacked PCP- or dextromethorphan-like discriminative-stimulus effects in rats (Holtzman 1994; Mori et al. 2001). Results with DTG have been mixed. Holtzman (1989) reported full substitution for PCP (at a 2.0 mg/kg training dose) in rats with others reporting no substitution (at a 1.25 mg/kg PCP training dose; Willetts and

[2]Full explanations and in-depth analyses of conditioning processes involved in the various behavioral procedures described in this chapter may be found in Catania (2013).

Table 2 Discriminative-stimulus effects of σR agonists (substitution for abused drugs and pretreatment)

Authors (Publication year)	Subject species	Training drug (dose in mg/kg, route)	Test drug (dose in mg/kg, route)	Outcomes (doses in mg/kg)
Cocaine				
Cohen and Sanger (1994)	Rat	Cocaine (6, i.p)	DTG (5), haloperidol (0.25), rimcazole (10), BMY 14802 (10)	No effects on cocaine DS: DTG Partial attenuation of cocaine DS: haloperidol Enhancement of cocaine DS: rimcazole, BMY 14802
Ukai et al. (1997)	Rat	Cocaine (10, i.p.)	DTG (1 or 10, s.c.)	No substitution Enhancement of cocaine DEC: DTG (10)
Katz et al. (2003)	Rat	Cocaine (10, i.p.)	Substitution (i.p.): rimcazole (3–30), SH 3-24 (0.3–17), SH 3-28 (3–17), GBR 12909 (3–100) Pretreatment (i.p.) against cocaine DEC (1–10, i.p.): rimcazole (5.6 and 10), SH 3-24 (5.6 and 10), SH 3-28 (5.6–17), GBR 12909 (10 and 17)	Full substitution: GBR 12909 No substitution: rimcazole, SH 3-24, SH 3-28 Dose-dependent enhancement of cocaine DEC: rimcazole, GBR 12909 No effects on cocaine DEC: SH 3-24, SH 3-28
Liu et al. (2007a)	Rat	Cocaine (10, i.p.)	Trishomocubanes, TC1 (10–100) and TC4 (3–30)	Partial substitution: TC1 (≤30% @ 56), TC4 (≤20% @ 3, 10 and 30)
Hiranita et al. (2011b)	Rat	Cocaine (10, i.p.)	PRE-084 (1–56, i.p.), DTG (0.1–17, i.p.), PRE-084 (1–32, s.c.), DTG (0.32–10, s.c.), PRE-084 (0.032–5.6, i.v.), DTG (0.32–5.6, i.v.), WIN 35,428 (0.1–3.2, i.p.), methylphenidate (0.32–3.2, i.p.)	Full substitution: WIN 35,428, methylphenidate No substitution: PRE-084 (i.p., s.c. or i.v.), DTG (i.p., s.c. or i.v.)
Matsumoto et al. (2011)	Rat	Cocaine (10, i.p.)	AC 927 substitution (1–10, i.p.) or pretreatment (3 and 5.6, i.p.)	Partial substitution: (≤40% @ 10) Dose-dependent enhancement of cocaine DEC

(continued)

Table 2 (continued)

Authors (Publication year)	Subject species	Training drug (dose in mg/kg, route)	Test drug (dose in mg/kg, route)	Outcomes (doses in mg/kg)
Rodvelt et al. (2011a)	Rat	Cocaine (5, i.p.)	SA4503 (0.3–30, i.p.)	Partial substitution at 3.0
			SA4503 (1, i.p.)	No effect on cocaine or *d*-amphetamine DEC
			SA4503 (0.3 and 1, i.p.)	Dose-dependent enhancement of *dl*-methamphetamine DEC
Methylphenidate				
Mori et al. (2014b)	Rat	Methylphenidate (5.0, i.p.)	Substitution: methamphetamine (0.125–1, s.c.), MDMA (0.625–2.5, s.c.), apomorphine (0.1–1, s.c.) Pretreatment against methylphenidate (0.625–5.0, s.c.) DEC: NE 100 (3, i.p.)	Full substitution: methamphetamine, No substitution: MDMA Partial substitution: apomorphine No effects on methylphenidate DEC: NE 100
d-Methamphetamine				
Witkin et al. (1993)	Rat	*d*-Methamphetamine (1, i.p.)	NPC 16377 (10 and 17, i.p.)	No substitution
Rodvelt et al. (2011b)	Rat	*dl*-Methamphetamine (0.5, s.c.)	SA4503 (0.3, 1, 3, i.p.)	Partial substitution No effect on cocaine or *d*-amphetamine DECs Upward shift in *dl*-methamphetamine DEC
MDMA				
Mori et al. (2014b)	Rat	MDMA (2.5, s.c.)	Substitution: (+)-pentazocine (0.3–10, i.p.), methamphetamine (0.25–2.0, s.c.), methylphenidate (1.25–5, i.p.) Pretreatment against MDMA (0.625–2.5, s.c.) DS: NE 100 (3, i.p.)	No substitution: methylphenidate, (+)-pentazocine, methamphetamine No effect on MDMA DS: NE 100
PCP				
Willetts and Balster (1988)	Rat	PCP (1.25, i.p.)	DTG (2–12, i.p.)	No substitution

(continued)

Table 2 (continued)

Authors (Publication year)	Subject species	Training drug (dose in mg/kg, route)	Test drug (dose in mg/kg, route)	Outcomes (doses in mg/kg)
Holtzman (1989)	Rat	PCP (2.0, i.p.)	DTG (0.25–4.0, s.c.)	Full substitution
Witkin et al. (1993)	Rat	PCP (1.5, i.p.) or (+)-MK 801 (0.2, i.p.)	NPC 16377 (1–17, i.p.)	No substitution
Mori et al. (2001)	Rat	PCP (1.5, i.p.)	Substitution (i.p.): (+)-pentazocine (10 and 30), cocaine (5.6–17), (+)-SKF-10,047 (0.3–10), (+)-MK 801 (0.01–0.2) Pretreatment (i.p.) against DS (i.p.) of PCP (1.5), (+)-MK 801 (0.2) and (+)-SKF-10047 (10): NE-100 (5), BMY-14802 {5 [PCP, (+)-MK 801 and (+)-SKF-10047] or 10 [only PCP]}	Full substitution: (+)-SKF-10,047, (+)-MK 801 No substitution: (+)-pentazocine Partial substitution: cocaine (≤30%) No effects on DS of PCP, (+)-MK 801 or (+)-SKF-10047: NE-100, BMY-14802
Ketamine				
Narita et al. (2001a)	Rat	Ketamine (5, i.p.)	Pretreatment against ketamine DEC (1.25–5, i.p.): NE 100 (1, i.p.) Pretreatment against ketamine DS (5, i.p.): NE 100 (0.3–3, i.p.)	Small rightward shift in ketamine DEC No effects of NE 100 on ketamine DS
Narita et al. (2001b)	Rat	Ketamine (5, i.p.)	DTG (0.3–3, s.c.), ketamine (1.25–5, i.p.), PCP (0.1–1, i.p.), (+)-MK 801 (0.003–0.030, i.p.)	Full substitution: PCP, (+)-MK 801 Partial substitution: DTG
Dextromethorphan				
Holtzman (1994)	Rat	Dextromethorphan (30, s.c.)	Substitution (i.p. or s.c.): DTG (0.3–10), (+)-pentazocine (0.3–10), (+)-SKF-10,047 (0.3–3), PCP (0.1–1), (+)-MK 801 (0.003–0.03), morphine (0.1–3.0) Pretreatment (i.p. or s.c.) against dextromethorphan DS (30, s.c.): DTG (0.3–10), (+)-pentazocine (0.3–10)	Full substitution: (+)-SKF-10,047, PCP, (+)-MK 801 No substitution: DTG, (+)-pentazocine, morphine No effects on dextromethorphan DS: DTG, (+)-pentazocine

DEC dose-effect curve, *DS* discriminative stimulus

Balster 1988), partial substitution for ketamine (Narita et al. 2001b), and a failure to substitute for dextromethorphan (Holtzman 1994).

Several papers have reported training discriminative-stimulus effects of σR ligands (Table 3). Steinfels et al. (1988) trained the discrimination of (+)-pentazocine in rats and found complete substitution with (+)-SKF-10,047. Additionally, PCP only partially substituted for (+)-pentazocine. In contrast, (+)-pentazocine and DTG failed to substitute for (+)-SKF-10,047 in squirrel monkeys, or dextromethorphan in rats (Balster 1989; Holtzman 1993, 1994; Singh et al. 1990). Holtzman (1989) reported discriminative-stimulus effects of DTG in rats, that was accompanied by full substitution with (+)-pentazocine, (+)-SKF-10,047, PCP or morphine. However, the mixed σR/dopamine receptor antagonist haloperidol was without effects on the discriminative-stimulus effects of DTG, suggesting that those effects of DTG were not pharmacologically specific to the σR system.

In summary, the drug-discrimination literature indicates that the interoceptive stimulus effects of psychomotor stimulants, and likely the mechanisms for those effects, do not primarily involve the σR system as evidenced by a lack of substitution by agonists and by the lack of blockade by σR antagonists. Further, the pharmacology of compounds acting at σRs is distinct from that of compounds acting at NMDA glutamate or dopamine receptors.

3 Place Conditioning

During experimental sessions using these procedures, subjects (typically rodents) are placed in a chamber most often with two compartments.[3] The two compartments in which the place conditioning occurs have different features such as flooring (grids or mesh), wall decorations (solid or stripes), or types of bedding. During "pre-conditioning" sessions, subjects are initially allowed to ambulate throughout the different compartments, and their allocation of time in each is recorded as baseline. During subsequent "conditioning sessions" the compound under study is injected and the subject is restricted to one compartment. Vehicle injections typically precede daily conditioning sessions in which the subject is confined to the alternate compartment. These sessions are conducted to affect a Pavlovian (classical) conditioning of the distinctive stimuli of the compartment with the drug effect. During a subsequent drug-free post-conditioning test, the subject is given access to both compartments as in pre-conditioning sessions, and time allocation in each is again recorded. An increase compared to preconditioning in time allocated to the drug-paired compartment is considered an indication of an effective place conditioning, with the distinctive features of the drug-paired compartment as the conditional stimuli and the drug effect as the unconditional

[3] In some instances, there is a third, middle, compartment in which subjects are placed at the start of sessions. Time spent in this starting compartment is not typically considered, as the critical variable is time spent in the drug-paired compartment.

Table 3 Discriminative-stimulus effects of σR agonists and antagonists

Authors (Publication year)	Subject species	Training drug (dose in mg/kg, route)	Test drug (dose in mg/kg, route)	Outcomes (dose in mg/kg)
(+)-Pentazocine				
Steinfels et al. (1988)	Rat	(+)-Pentazocine (2.0, s.c.)	Substitution (s.c.): (+)-pentazocine (0.02–2.0), (+)-SKF-10,047 (0.04–1.0), PCP (0.04–1.0)	Full substitution: (+)-pentazocine, (+)-SKF-10,047 Partial substitution: PCP (≤50% @ 2.0)
DTG				
Holtzman (1989)	Rat	DTG (3.0, s.c.)	Substitution: DTG (0.25–4, s.c.), haloperidol (0.01–0.3, s.c.), (+)-pentazocine (0.3–30, s.c.), *d*-amphetamine (0.1–3.0, s.c.), (+)-SKF-10,047 (0.03–1, s.c.), PCP (0.1–3.0, i.p.), TCP (0.03–0.56, s.c.), morphine (0.1–10, s.c.), pentobarbital (1.0–17.5, s.c.) Pretreatment against DTG DS (3, s.c.): haloperidol (0.01–0.3, s.c.)	Full substitution (ED_{50}, 95%CL): DTG (0.48, 0.42–0.54), (+)-pentazocine (6.0, 5.5–6.5), (+)-SKF-10,047 (0.15, 0.12–0.18), PCP (0.35, 0.31–0.39), morphine (0.83, 0.72–0.94) No substitution: haloperidol, *d*-amphetamine, pentobarbital Partial substitution: TCP (≤80%) No effect on DTG DS (3 mg/kg): haloperidol
(+)-SKF-10,047				
Balster (1989)	Rat	(+)-SKF-10,047 (10, i.p.)	Substitution (i.p.) and pretreatment (i.p.) against (+)-SKF-10,047 DS (10, i.p.): haloperidol (0.006–0.4)	No substitution and no effects on (+)-SKF-10,047 DS: haloperidol
		(+)-SKF-10,047 (5, i.p.)	Substitution (i.p.): DTG (0.5–8), (+)-SKF-10,047 (1.7–30), PCP (0.1–10), ketamine (1–30), Pretreatment (i.p.) with (+)-SKF-10,047 (5, i.p.): DTG (0.5–8)	Full substitution [ED_{50}, 95%CL (μmol/kg)]: (+)-SKF-10,047 [11.1 (8.7–14.1)], PCP [1.9 (1.4–2.5)], ketamine [12.2 (9.1–16.5)], No substitution: DTG No effects on (+)-SKF-10,047 DS: DTG,

(continued)

Table 3 (continued)

Authors (Publication year)	Subject species	Training drug (dose in mg/kg, route)	Test drug (dose in mg/kg, route)	Outcomes (dose in mg/kg)
Singh et al. (1990)	Rat	(+)-SKF-10,047 (3.0, s.c.)	Substitution (s.c. unless noted): DTG (3–30), *d*-amphetamine (0.25–1.0), *R*(−)-apomorphine (0.075–0.5), (+)-SKF-10,047 (0.8–3.0), PCP (0.25–2.0), (+)-MK-801 (0.075–0.5, i.p.) Pretreatment (s.c.) against (+)-SKF-10,047 DS (3.0, s.c.): DTG (3–30), haloperidol (0.025–0.4), BMY-14802 (1–30)	Full substitution: (+)-SKF-10,047, PCP, (+)-MK-801 No substitution: DTG, *d*-amphetamine, *R*(−)-apomorphine No effects on (+)-SKF-10,047 DS: DTG, haloperidol, BMY-14802
Holtzman (1993)	Squirrel monkey	(+)-SKF-10,047 (1.0, i.m.)	Substitution (i.m.): (+)-pentazocine (1.0–4.0), DTG (1.0–8.0), haloperidol (0.008–0.064), BMY 14802 (0.25–4.0), (+)-SKF-10,047 (0.25–2.0), TCP (0.008–0.032), PCP (0.032–0.205), (+)-MK-801 (0.0005–0.004), morphine (0.25–4.0) Pretreatment (i.m.) against (+)-SKF-10,047 (1.0, i.m.) DS: haloperidol (0.008–0.064), BMY 14802 (0.64–4.0) Pretreatment (i.m.) against PCP DS (0.25, i.m.): haloperidol (0.008–0.064), BMY 14802 (0.25–2.0)	Full substitution (ED_{50}, 95%CL): (+)-SKF-10,047 (0.50, 0.40–0.62), TCP (0.016, 0.010–0.028), PCP (0.089, 0.072–0.110) No substitution: (+)-pentazocine, DTG, haloperidol, BMY 14802, morphine Partial substitution: (+)-MK-801 ($\leq$72%) Partial attenuation of (+)-SKF-10,047 DS: haloperidol, BMY 14802 No effects on PCP DS: haloperidol, BMY 14802

(continued)

Table 3 (continued)

Authors (Publication year)	Subject species	Training drug (dose in mg/kg, route)	Test drug (dose in mg/kg, route)	Outcomes (dose in mg/kg)
Pregnanolone				
Engel et al. (2001)	Rat	Pregnanolone (5, i.p.)	All i.p.: (+)-SKF-10,047 (5–20), morphine (1–9), pregnanolone (1–10), pentobarbital (1–10), midazolam (0.1–5), ethanol (500–2,000), (+)-MK 801 (0.05–0.2)	Full substitution (ED50, 95%CL): (+)-SKF-10,047 (9.7, 6.0–15.6), pentobarbital (2.9, 2.2–3.8), pregnanolone (1.7, 1.3–2.2), midazolam (0.5, 0.2–1.2), ethanol (900, 700–1,100), Partial substitution: morphine, (+)-MK 801
Dextromethorphan				
Holtzman (1994)	Rat	Dextromethorphan (30, s.c.)	Substitution (i.p. or s.c.): DTG (0.3–10), (+)-pentazocine (0.3–10), (+)-SKF-10,047 (0.3–3), PCP (0.1–1), (+)-MK 801 (0.003–0.03), morphine (0.1–3.0) Pretreatment (i.p. or s.c.) against dextromethorphan DS (30, s.c.): DTG (0.3–10), (+)-pentazocine (0.3–10)	Full substitution (ED50, 95%CL): (+)-SKF-10,047 (2.05, 1.50–2.81), PCP (0.34, 0.17–0.68), (+)-MK 801 (0.013, 0.007–0.024) No substitution: DTG, (+)-pentazocine, morphine No effects on dextromethorphan DS: DTG, (+)-pentazocine
BMY 14802				
Vanecek et al. (1998)	Pigeon	BMY 14802 (5.6, i.m.)	Substitution (i.m.): DTG (1.0–18), haloperidol (0.032–3.2), rimcazole (1.8–100), BMY-14802 (0.32–32), (+)-SKF-10,047 (0.1–18), PCP (0.1–10) Pretreatment (i.m.) against BMY-14802 DEC (0.32–10, i.p.): DTG (5.6)	Full substitution (ED50, 95%CL): BMY-14802 (1.5, 1.0–2.1) No substitution: DTG, haloperidol, rimcazole, (+)-SKF-10,047, PCP, No effects on BMY 14802 DEC: DTG

TCP *N*-[1-(2-thienyl)cyclohexyl]-piperidine, *95%CL* 95% confidence limit. *DS* discriminative stimulus, *ED50* dose that produces a 50% effect or an effect in 50% of the population

stimulus. Allocation of greater time in post- compared to pre-conditioning may be considered a result of conditioned reinforcing effects of the conditional stimulus which results in the increase in time allocation. Those increases in time allocation are thought to provide an indication of the reinforcing effects and potential for abuse of the tested compound. Alternatively, a decrease in time allocated to the drug-paired compartment is also considered an indication of an effective place conditioning, but rather a noxious effect of the conditional stimuli due to an unconditional noxious effect of the drug. Assessments of the literature utilizing this procedure have indicated that it has reasonable predictive validity for drugs of abuse, though it has its share of false positives and negatives (e.g., Bardo and Bevins 2000; Carr et al. 1989).

Several studies have examined place conditioning with σR ligands (Table 4). Most of these experiments were conducted as controls for studies of the potential antagonism by the σR ligands of the place conditioning produced by drugs of abuse. Consequently, the dose ranges examined are often more restricted than those desirable for a full assessment of the effects of the σR ligands. Nonetheless, none of the studies conducted to date have demonstrated place conditioning with any of the σR ligands, agonists or antagonists, when administered alone in naïve rodents (Table 4). However, it appears that cocaine exposure can render σR agonists active in vivo. For example, a study using a reinstatement model reported that σR agonists, igmesine and dehydroepiandrosterone (DHEA), alone reinstated extinguished place preference for cocaine (Romieu et al. 2004). This curious result is an augury of later findings using drug self-administration procedures (see below).

The extensive studies conducted by T. Maurice and colleagues were the first studies reporting antagonism of stimulant-induced place conditioning in mice (Romieu et al. 2000, 2002, 2003, 2004). In those studies (Table 5), the σR ligands were administered in combination with cocaine, either 10 or 20 mg/kg, before conditioning sessions. As reported, the σR antagonists, NE 100, BD1047, and progesterone blocked acquisition of cocaine-induced place conditioning. Additionally, the same compounds blocked the "expression" of place conditioning when administered only during the post-conditioning test session. Finally, knock-down of σ_1Rs with centrally administered σ_1R antisense oligodeoxynucleotide inhibited the development of cocaine-induced place conditioning, whereas antisense mismatch controls exhibited place conditioning (Romieu et al. 2000). Several subsequent studies replicated these effects on place conditioning with cocaine in mice using other σR antagonists such as the σ_1 preferential, AC 927 (Matsumoto et al. 2011), and YZ-185 (Sage et al. 2013), or conditioning with methamphetamine (Rahmadi et al. 2013).

Interestingly, the σ antagonist, CM 156, was inactive when administered with cocaine in mice, i.e. it failed to block the place conditioning induced by cocaine (Xu et al. 2010). However, when administered alone during the post-conditioning test it blocked the "expression" of place conditioning (Table 6). In a follow-up study (Xu et al. 2012) the mouse brain tissues were collected immediately after the

Table 4 Place conditioning with σR Ligands

Authors (Publication year)	Subject species	Conditioning drug (dose in mg/kg unless noted, route)	Test drug (dose, route)	Outcomes
Agonists				
Nam et al. (2012)	Mouse	Dextromethorphan (35, i.p.)	BD1047 (1 and 2, i.p.), SM 21 (5 and 10, i.p.)	Neither compound induced place conditioning or aversion
Romieu et al. (2003)	Mouse	DHEA (5, 10, and 20, s.c.), Igmesine (10 and 30 mg/kg, i.p.), Pregnenolone (10, and 20, s.c.)	None	DHEA, igmesine, or pregnenolone (agonists) did not induce place conditioning or aversion
Romieu et al. (2002)	Mouse	Igmesine (10, 30, and 60, i.p.) PRE-084 (10, 30, and 60, i.p.)	None	Igmesine or PRE-084 did not induce place conditioning or aversion
Bhutada et al. (2012)	Mouse	PRE-084 (0.001–10 μg/mouse, i.c.v.)	None	PRE-084 did not induce place conditioning or aversion
Mori et al. (2014a)	Rat	SA4503 (0.3–3, s.c.)	None	SA4503 did not induce place conditioning or aversion
Horan et al. (2001)	Rat	SA4503 (1 and 3, i.p.)	None	SA4503 did not induce place conditioning or aversion
Antagonists				
Matsumoto et al. (2011)	Mouse	AC 927 (5 and 10, i.p.)	None	AC 927 did not induce significant place conditioning or aversion, though there was a non-significant trend towards place aversion
Bhutada et al. (2012)	Mouse	BD1047 (0.1–10 μg/mouse, i.c.v.)	None	BD1047 did not induce place conditioning or aversion
Chen et al. (2011)	Rat	BD1047 (3, i.p.)	None	BD1047 did not induce place conditioning or aversion
Romieu et al. (2000)	Mouse	BD1047 (1, 3, and 10, i.p.) NE 100 (1, 3, and 10, i.p.)	None	NE 100 or BD1047 did not induce place conditioning or aversion
Romieu et al. (2003)	Mouse	BD1047 (10, i.p.), Progesterone (10, 20, and 40, s.c.), Finasteride (25, b.i.d., a 5α-reductase inhibitor)	None	BD1047, progesterone or finasteride did not induce place conditioning or aversion

(continued)

Table 4 (continued)

Authors (Publication year)	Subject species	Conditioning drug (dose in mg/kg unless noted, route)	Test drug (dose, route)	Outcomes
Nam et al. (2012)	Mouse	BD1047 (1 and 2, i.p.), SM 21 (5 and 10, i.p.)	None	BD1047 and SM 21 did not induce place conditioning or aversion
Mori et al. (2012)	Rat	NE 100 (0.3 and 1, i.p.)	None	NE 100 did not induce place conditioning or aversion
Sage et al. (2013)	Mouse	YZ-185 (0.042, 3.16, and 31.6, unspecified route of administration)	None	YZ-185 did not induce place conditioning or aversion

behavioral assessments. Alterations in gene expression were determined by cDNA microarray analysis with four genes (metastasis associated lung adenocarcinoma transcript 1, tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, and transthyretin) involved in processes related to neuro-adaptive change and RNA editing. Each was consistently upregulated by cocaine when compared to saline controls. Those upregulations were also found to be reversed by CM 156, with quantitative real time PCR confirming the reversal in three of the four genes. The results suggest that σR antagonism can reverse relatively long-term molecular changes associated with stimulant reinforcement.

A previous study by Romieu et al. (2002) found that the σR antagonists, NE100 and BD1047, blocked both place conditioning and the expression of place conditioning induced by cocaine in mice. However, the σR antagonist, YZ-185, blocked the development of cocaine place conditioning in mice, but not its expression (Sage et al. 2013).

There are fewer studies of the effects of σR agonists on stimulant-induced place conditioning. Romieu et al. (2003) found that DHEA, igmesine, and pregnenolone enhanced cocaine place conditioning in mice, and that the enhancement was blocked by BD1047. However, Mori et al. (2014a) found that the σR agonist, SA4503 (1 and 3 mg/kg), attenuated acquisition of place conditioning induced by cocaine, methamphetamine, and morphine in rats, whereas (+)-pentazocine was inactive, which begs the question of whether the effect was mediated by σRs, or whether the 3.0 mg/kg dose of (+)-pentazocine was too low to enhance the place conditioning.

In summary, the findings from studies of place conditioning have several consistencies. One of these is that neither σR agonists nor antagonists have effects of their own as unconditional stimuli. Further, the σR antagonists appear to consistently block the acquisition of place conditioning with stimulants. However, the effects of σR agonists are less consistent. This is not the only instance in which σR agonists and antagonists do not have diametrically opposed effects. The effects of CM 156 on the expression of an already developed place conditioning and

Table 5 Antagonism by σR ligands of acquisition of stimulant place conditioning

Authors (Publication Year)	Subject species	Conditioning drug (dose in mg/kg/day, route)	Test drug (dose in mg/kg/day, route)	Outcomes (dose where indicated is in mg/kg/day)
Cocaine				
Romieu et al. (2000)	Mouse	Cocaine (20 for 4 days, i.p.)	BD1047 (1, 3, and 10 for 4 days, i.p.), NE 100 (1, 3, and 10 for 4 days, i.p.), σ_1R antisense (i.c.v.)	NE 100, BD1047, or σ_1R antisense blocked cocaine place conditioning
Romieu et al. (2002)	Mouse	Cocaine (20 for 4 days, i.p.)	BD1047 (1, 3, and 10 for 4 days, i.p.), NE 100 (1, 3, and 10 for 4 days, i.p.)	NE 100 or BD1047 blocked cocaine place conditioning
		BTCP (10 for 4 days, i.p.)	BD1047 (1, and 3 for 4 days, i.p.), NE 100 (3, and 10 for 4 days, i.p.)	NE 100 or BD1047 blocked BTCP place conditioning
Romieu et al. (2003)	Mouse	Cocaine (10 for 4 days, i.p. or s.c.)	DHEA (5, 10, and 20, s.c.), igmesine (10, i.p.), pregnenolone (10, and 20, s.c.), BD1047 (10, i.p.), progesterone (20, i.p.)	DHEA, igmesine, and pregnenolone enhanced cocaine place conditioning BD1047 blocked cocaine place conditioning and its enhancement by the steroidal agonists Progesterone blocked cocaine place conditioning and its enhancement by igmesine
		Cocaine (20 for 4 days, i.p. or s.c.)	Progesterone (10, 20, and 40, s.c.), finasteride (25 mg/kg, b.i.d. for 6 days)	Progesterone (but not 40) and finasteride blocked cocaine place conditioning. Progesterone (40) partially but seemingly blocked acquisition of cocaine place conditioning

(continued)

Table 5 (continued)

Authors (Publication Year)	Subject species	Conditioning drug (dose in mg/kg/day, route)	Test drug (dose in mg/kg/day, route)	Outcomes (dose where indicated is in mg/kg/day)
Romieu et al. (2004)	Mouse	Cocaine (30 for 4 days, i.p.)	Igmesine (1, 3, and 10 mg/kg, i.p.), DHEA (10, 20, and 40 mg/kg, s.c.), BD1047 (3, 10, and 30 mg/kg, i.p.) σ_1 antisense (i.c.v.)	Reinstatement of place conditioning with: 1) Cocaine (15 mg/kg, i.p.) which was blocked by BD1047 or σ_1 antisense 2) Igmesine (10 mg/kg, i.p.) and DHEA (40 mg/kg, s.c.) which was blocked by BD1047 (30 mg/kg, i.p.) 3) PCP (5 mg/kg, i.p.), morphine (5 mg/kg, i.p.), and nicotine (0.5 mg/kg, i.p.) which was blocked by BD1047 (30 mg/kg, i.p.) 4) Ethanol (1,000, i.p.) was enhanced by BD1047 (30 mg/kg, i.p.) Igmesine and DHEA alone reinstated cocaine place conditioning BD1047 (30 mg/kg, i.p.) alone did not reinstate cocaine place conditioning
Matsumoto et al. (2011)	Mouse	Cocaine (10 and 20 for 4 days, i.p.)	AC 927 (5 and 10 for 4 days, i.p.)	AC 927 attenuated cocaine place conditioning [Conditioning with AC 927 (5 and 10) produced a non-significant trend suggesting noxious effects.]
Xu et al. (2012)	Mouse	Cocaine (20 for 4 days, i.p.)	CM 156 (1–20, i.p.)	CM 156 failed to block cocaine place conditioning
Sage et al. (2013)	Mouse	Cocaine (20 for 3 days)	YZ-185 (0.042, 1.31, and 13.1 for 3 days)	YZ-185 (13.1) fully blocked cocaine place conditioning

(continued)

Table 5 (continued)

Authors (Publication Year)	Subject species	Conditioning drug (dose in mg/kg/day, route)	Test drug (dose in mg/kg/day, route)	Outcomes (dose where indicated is in mg/kg/day)
Mori et al. (2014a)	Rat	Cocaine (4 for 3 days, i.p.)	SA4503 (1 and 3 for 3 days, s.c.), (+)-pentazocine (3 for 3 days, s.c.)	SA4503 dose-dependently but partially attenuated place conditioning of cocaine (+)-pentazocine did not block place conditioning of cocaine
Dextromethorphan				
Nam et al. (2012)	Mouse	Dextromethorphan (35 for 14 days, i.p.)	BD1047 (1 and 2 for 14 days, i.p.), SM 21 (5 and 10 for 14 days, i.p.)	BD1047 partially attenuated dextromethorphan place conditioning SM 21 was without effects on dextromethorphan place conditioning
Methamphetamine				
Mori et al. (2014a)	Rat	Methamphetamine (2 for 3 days, i.p.)	SA4503 (1 and 3 for 3 days, s.c.), (+)-pentazocine (3 for 3 days, s.c.)	SA4503 partially attenuated place conditioning of methamphetamine (+)-Pentazocine did not block place conditioning of methamphetamine
Rahmadi et al. (2013)	Mouse	Methamphetamine (1 for 3 days, s.c.)	Fluoxetine (20 for 3 days, i.p.), NE 100 (1 for 3 days, i.p.)	NE 100 fully reversed a fluoxetine-induced decrease in methamphetamine place conditioning
PCP				
Nabeshima et al. (1996) *Original source: Kitaichi et al. (1995)	Rat	PCP (4 for 3 days, i.p.)	NE 100 (0.03–0.1 for 3 days, p.o.)	NE-100 did not block PCP conditioned aversion

BTCP benzothiophenylcyclohexylpiperidine

associated long-term changes in gene expression show promise for examining how σRs may contribute to chronic stimulant abuse, and hopefully the inconsistencies among outcomes with different compounds might be resolved with the examination of a wider dose range and more mechanistic studies.

Table 6 Antagonism of expression of place conditioning with σR ligands

Authors (Publication year)	Subject species	Conditioning drug (dose in mg/kg/day, route)	Test drug (dose in mg/kg, route)	Outcomes (dose in mg/kg)
Cocaine				
Romieu et al. (2002)	Mouse	Cocaine (20 for 4 days, i.p.)	BD1047 (1, 3, and 10, i.p.), NE 100 (3 and 10, i.p.)	NE 100 or BD1047 blocked cocaine place conditioning
Romieu et al. (2004)	Mouse	Cocaine (30 for 4 days, i.p.)	Igmesine (1, 3, and 10, i.p.), DHEA (10, 20, and 40, s.c.), BD1047 (3, 10, and 30, i.p.), σ_1R antisense	Reinstatement of place conditioning with: 1) Cocaine (15, i.p.) which was blocked by BD1047 or σ_1R antisense 2) Igmesine (10, i.p.) and DHEA (40, s.c.) which was blocked by BD1047 (30, i.p.) 3) PCP (5, i.p.), morphine (5, i.p.), and nicotine (0.5, i.p.) which was blocked by BD1047 (30, i.p.) 4) Ethanol (1,000, i.p.) was enhanced by BD1047 (30, i.p.) Igmesine and DHEA alone reinstated cocaine place conditioning BD1047 (30, i.p.) alone did not reinstate cocaine place conditioning
Xu et al. (2010)	Mouse	Cocaine (20 for 4 days, i.p.)	CM 156 (1–20, i.p.)	CM 156 dose-dependently but partially blocked expression of cocaine place conditioning
Xu et al. (2012)	Mouse	Cocaine (20 for 4 days, i.p.)	CM 156 (1–20, i.p.)	CM 156 dose-dependently but partially blocked expression of cocaine place conditioning
Sage et al. (2013)	Mouse	Cocaine (20 for 3 days, unspecified route of administration)	YZ-185 (0.042, 1.31, and 13.1, unspecified route of administration)	YZ-185 did not block cocaine place conditioning
Fritz et al. (2011)	Rat	Cocaine (15 for 4 days, i.p., unspecified regarding bias)	BD1047 (10, i.p.)	BD1047 fully blocked cocaine place conditioning

4 Drug Self-Administration

In procedures of this type an easily repeatable response produces an intravenous drug injection through a chronic indwelling catheter. The rate at which the subject responds is recorded and is compared to the rate of response when vehicle is substituted for the stimulant. This procedure is generally thought to be the "gold standard" for assessments of the abuse liability of compounds, and is often used to assess the effects of potential medical treatments for drug abuse (see reviews by Grabowski et al. 2004; Horton et al. 2013; O'Connor et al. 2011).

Because σR agonists were at one point believed to be in a similar pharmacological class with PCP, the earliest studies examining self-administration of σR ligands compared them to PCP. In one of the first of these (Slifer and Balster 1983), enantiomers of the original prototype σR agonist, SKF-10,047, were compared with PCP for their reinforcing effects in rhesus monkeys trained to self-administer cocaine (Fig. 1). Previous studies had demonstrated that the racemic SKF-10,047 failed to substitute for ketamine or codeine in rhesus monkeys (Young and Woods 1981), though stereoselectivity of the σR specific effects of SKF-10,047 was yet to be demonstrated (e.g. Brady et al. 1982; Su 1982). In the paper by Slifer and Balster (1983), neither the racemate nor the (−)-enantiomer of SKF-10,047 (which has kappa agonist opioid effects (Martin et al. 1984; Picker and Dykstra 1987; Slifer

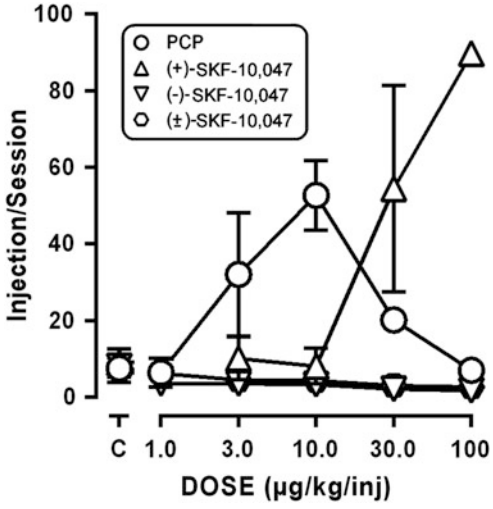

Fig. 1 Self-administration of PCP, (+)-SKF-10,047, (−)-SKF-10,047, or (±)-SKF-10,047 when substituted for cocaine in male rhesus monkeys trained to self-administer cocaine (50 µg/kg/injection). Ordinates: Injections per session. Abscissae: Drug dose in µg/kg/injection, log scale. Each point represents the mean (±SEM) of three subjects. Symbols above C represent injections/session when vehicle was substituted for cocaine. Note that only PCP and (+)-SKF-10,047 maintained self-administration responding above vehicle levels. Adapted from Slifer and Balster (1983) and used with permission from RL Balster

and Dykstra 1987)) substituted for cocaine, whereas the (+)-enantiomer did substitute for cocaine. At the time of the study a specific σR had not been identified, and a similarity of the self-administration of PCP suggested that SKF-10,047 had PCP-like pharmacology. And indeed even when a σR had been identified, it was established that (+)-SKF-10,047 had affinity for both σRs and, as with PCP, the NMDA binding site (Chou et al. 1999; Lever et al. 2016; Shin et al. 2005).

σR Agonist Self-Administration In the course of studies examining the effects of σR antagonists on cocaine self-administration (described below) in rats, the effects of the σR agonists, DTG and PRE-084, were also studied (Hiranita et al. 2010). Surprisingly, each of these compounds produced a dose-related enhancement of cocaine self-administration, evidenced by a leftward shift in the cocaine dose-effect curve. This shift was similar to the effects of dopamine uptake inhibitors on cocaine self-administration (Barrett et al. 2004; Hiranita et al. 2009; Schenk 2002). As dopamine uptake inhibitors are themselves self-administered, subsequent studies assessed whether σR agonists would be self-administered. In those studies, subjects with a history of cocaine self-administration readily self-administered the σR receptor agonists, DTG and PRE-084 (Hiranita et al. 2010).

The reinforcing effects of the σR agonists in cocaine-experienced subjects posed the question of whether these compounds would be self-administered in experimentally naïve subjects (Hiranita et al. 2013a). Using a dose of PRE-084 that was self-administered at the highest rate in the previous study (Hiranita et al. 2010), as well as (+)-pentazocine in a second group of rats, the σ_1R agonists were made available for self-administration in experimentally naïve rats. Over the course of 28 daily experimental sessions, responses on one of two available levers produced a σ_1R agonist injection, either PRE-084 or (+)-pentazocine in the separate groups of rats. Responses on the alternate lever had no scheduled consequences. The 28 sessions of σ_1R agonist availability is about three-fold greater than the number sufficient for the acquisition of cocaine self-administration under these same conditions. Nonetheless, over the course of those sessions there was no appreciable self-administration of either σ_1R agonist. Further, doses of PRE-084 ranging from 0.1 to 10.0 mg/kg/inj were similarly inactive (Hiranita et al. 2013a).

The same subjects were subsequently allowed to self-administer cocaine (0.32 mg/kg/inj) over the course of 14 daily sessions. During those sessions acquisition of cocaine self-administration was obtained with each response producing a cocaine injection. After acquisition of cocaine self-administration, previously inactive doses of both PRE-084 or (+)-pentazocine (each at 0.32 mg/kg/inj) were in different groups of rats substituted for cocaine. During the subsequent ten consecutive sessions, responding was well-maintained by either σ_1R agonist on the same (right) lever on which responses previously produced cocaine injections (Fig. 2a, b). Over the course of the next seven sessions, each response on the left lever, which previously had no effect, now produced injections of the σ_1R agonists, with

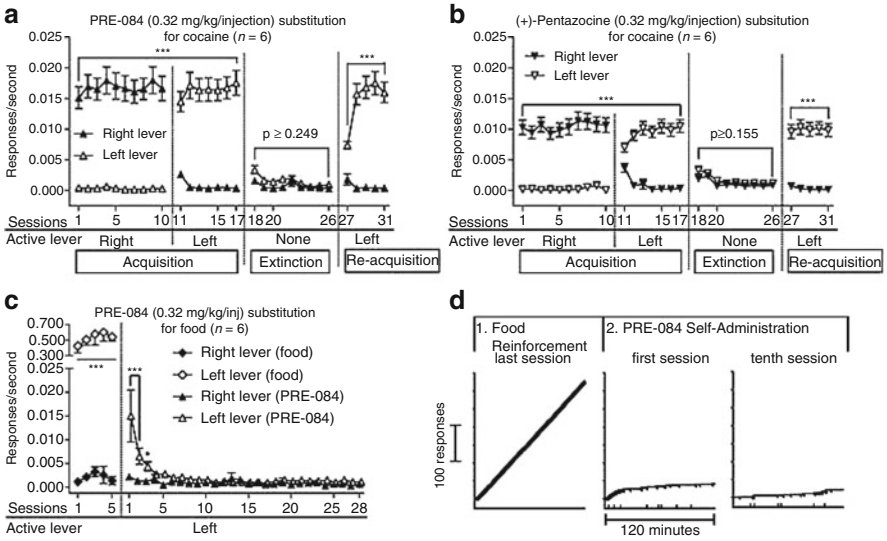

Fig. 2 Selective σ_1R agonist self-administration after cocaine experience, but not after experience with food reinforcement. Each point represents the mean (±SEM) of six subjects. (**a**, **b**) Self-administration of selective σ_1R agonists PRE-084 or (+)-pentazocine when each response produced an injection. Ordinates: Responses per sec. Abscissae: Sequential daily sessions. Reversal of active and inactive levers, extinction, and reacquisition each had the effects expected for a reinforcing agent (see text for an explanation). (**c**) A history of consistent responding maintained by food reinforcement was not sufficient to induce reinforcing effects of PRE-084. Ordinates: Responses per sec. Abscissae: Sequential daily sessions. (**d**) Cumulative responses of a representative subject in real time. Abscissae: elapsed time during experimental sessions. Ordinates: cumulative responses emitted. Each food presentation or PRE-084 injection produced a diagonal slash mark on the record. The first record is from the last session of responding maintained by food presentation; responding was so frequent that the slash marks are contiguous and render the cumulative record as a *thick line*. The second record is from the immediately following session, which was the first session in which there was an opportunity to self-administer PRE-084 (0.32 mg/kg/injection), conducted the day following the last session with food reinforcement. This record shows the extinction of responding previously maintained by food reinforcement despite response-dependent PRE-084 injections. The record from the tenth session confirms the absence of acquisition of PRE-084 self-administration. Statistical significance levels are indicated where appropriate as $p < 0.001$ (***) or $p < 0.05$ (*) as compared with responding on the inactive lever (post-hoc Bonferroni *t*-test). Adapted from Hiranita et al. (2013a)

responses on the right lever no longer having effects (extinction, EXT). During the first of these sessions, subjects switched to responding on the left, newly active, lever and responding on the previously active, right, lever was virtually eliminated (Fig. 2a, b). Subsequently responses on neither of the levers had consequences and responding decreased to low rates on both. Finally, σ_1R agonist injections were again made available for responses on one of the levers and responding was promptly re-established (Fig. 2a, b; Hiranita et al. 2013a).

A similar study was conducted with food-reinforced responding in rats (Hiranita et al. 2013a). After lever-press training with each response producing a food pellet, surgical catheterization, recovery, and another five daily sessions of food reinforcement, PRE-084 (0.32 mg/kg/inj) replaced food presentations as the consequence for responding. Over the next five daily sessions, response rates progressively declined to low rates (Fig. 2c). Cumulative records of responding (Fig. 2d) show the constant high rate of responding during the last session of food reinforcement, a negatively accelerated temporal pattern of responding during the first session of PRE-084 self-administration characteristic of extinction (e.g., Catania 2013), and the low overall rate of occasional responding that occurred for the remainder of the 28 daily sessions of PRE-084 self-administration. These results indicate that the self-administration of PRE-084 after cocaine self-administration was not simply due to high persistent rates of operant responding per se, regardless of the consequence, which continued once the schedule of σ_1R agonist self-administration was superimposed. Additionally, as the subjects received relatively large numbers of PRE-084 injections within the first several sessions when substituted for food (Fig. 2c, d), its lack of self-administration after food reinforcement was not likely due to inadequate exposure to the contingency between responses and consequent PRE-084 injection.

The generality of the induction of σ_1R agonist self-administration was assessed in an additional study (Hiranita et al. 2013b). Rats were trained to self-administer the dopamine releaser, *d*-methamphetamine (0.1 mg/kg/inj), the mu-opioid receptor agonist, heroin (0.01 mg/kg/inj), and the non-competitive NMDA receptor/channel antagonist ketamine (0.32 mg/kg/inj). Each of the doses used was one that produced maximal rates of self-administration in previous studies. As with cocaine, self-administration of *d*-methamphetamine induced reinforcing effects of PRE-084 and (+)-pentazocine (0.032–1.0 mg/kg/inj, each). In contrast, neither self-administration of heroin nor ketamine induced σ_1R agonist self-administration (0.032–10 mg/kg/inj, each). Though the σ_1R agonists did not maintain responding in subjects with histories of heroin or ketamine self-administration, substitution for those drugs was obtained with other compounds; remifentanil substituted for heroin and (+)-MK 801 substituted for ketamine (Hiranita et al. 2013b).

The pharmacological mechanisms involved in the self-administration of cocaine were contrasted with those for σ_1R agonist self-administration in studies with antagonists (Hiranita et al. 2013b). Rats were trained under a fixed-ratio (FR) 5-response schedule of cocaine self-administration (each fifth response produced an injection) with doses increasing across components of the daily experimental sessions. In the first component responses had no scheduled consequences (extinction or EXT), whereas in subsequent components, separated by 2-min "timeout" periods with all chamber lights off and no scheduled consequences for responses, the dose/injection was increased from 0.03 to 1.0 mg/kg. Details of the procedure or similar ones have been published elsewhere (Barrett et al. 2004; Hiranita et al. 2009; Schenk 2002).

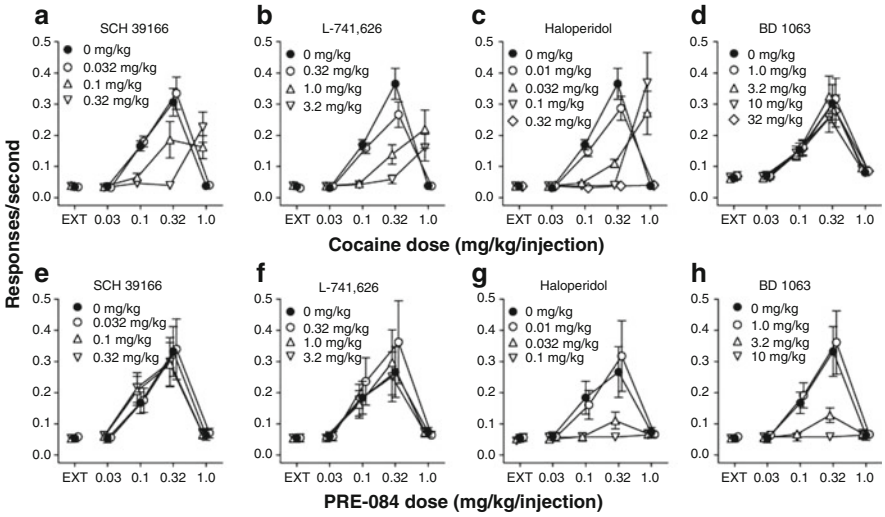

Fig. 3 Effects of antagonists on cocaine and PRE-084 self-administration. Ordinates: Responses per sec. Abscissae: Cocaine or PRE-084 injection dose in mg/kg, log scale. Rats ($N = 6$) were trained to self-administer cocaine under a fixed-ratio (FR) five-response schedule of reinforcement. Sessions were divided into five 20-min components with different doses of cocaine available in successive components. Consequences of responding in the successive components were: 0 mg/kg (no injection or "extinction," EXT) or, in the subsequent four components, doses of 0.032–1.0 mg/kg/injection. All antagonists were administered intraperitoneally (i.p.), 30 or 5 min (BD1063) before sessions. Each point represents the mean (±SEM) of response rates on the active of two levers in the chamber. (**a**) Effects of SCH 39166, a selective dopamine D_1-like receptor antagonist; (**b**) Effects of L-741,626, a selective dopamine D_2-like receptor antagonist. (**c**) Effects of haloperidol, a non-selective σ/dopamine receptor antagonist. Each of the dopamine antagonists produced a dose-related rightward shift in the cocaine self-administration dose-effect curve. (**d**) The preferential σ_1R antagonist, BD1063, did not substantially affect cocaine self-administration. (**e**, **f**) The selective dopamine antagonists did not substantially affect PRE-084 self-administration. (**g**, **h**) Both haloperidol and BD1063 dose-dependently decreased maximal PRE-084 self-administration. Adapted from Hiranita et al. (2013a)

The dopamine D_1-like receptor antagonist, SCH 39166, shifted the cocaine self-administration dose-effect curve to the right in a dose-related manner (Fig. 3a). Similar effects were obtained with the dopamine D_2 receptor preferential antagonist, L-741,626 (Fig. 3b). The non-selective dopamine antagonist haloperidol also produced dose-related rightward shifts in the cocaine self-administration dose-effect curve with its highest dose producing an insurmountable antagonism across the cocaine doses studied (Fig. 3c). In contrast, the σR antagonist BD1063 was inactive against cocaine self-administration over a range of doses from 1 to 32 mg/kg (Fig. 3d).

In these same subjects PRE-084 was occasionally substituted for cocaine, with and without antagonist pretreatments. In contrast to the antagonism of cocaine self-administration, the dopamine antagonists, SCH 39166, and L-741,626 (Fig. 3e, f), were inactive against PRE-084 self-administration. In contrast, both haloperidol

(Fig. 3g) and BD1063 (Fig. 3h) blocked the self-administration of PRE-084 in a dose-related manner. Both of these compounds have been reported to possess σR antagonist effects (de Costa et al. 1993; Hayashi and Su 2007). Taken together, the antagonism studies suggest that self-administration of σ_1R agonists is independent of dopamine systems, distinguishing it from stimulant and opioid agonist self-administration.

The difference in the blockade of cocaine and PRE-084 self-administration deserves some further discussion. As most effectively demonstrated with haloperidol the antagonism of cocaine self-administration can be characterized as a rightward shift in its dose-effect curve. In contrast, the effects of haloperidol on PRE-084 self-administration can be characterized as a dose-dependent decrease in the maximal effect. There is currently no adequate explanation of these differences. However, the bitonic nature of the cocaine self-administration dose-effect curve may provide some direction. With certainty multiple and differing behavioral and pharmacological mechanisms contribute to the two limbs of the self-administration dose-effect curve (see Katz 1989; Woods et al. 1987; Zernig et al. 2007 for a description). One hypothetical explanation would derive from a minimal sensitivity to σR antagonism of the mechanisms contributing to the descending limb of the curve. A good example of a pharmacological analysis of another bitonic curve has been published (Collins et al. 2005). That analysis maps out a clear way to proceed, though at this juncture, mechanisms for the descending limb of the σ_1R agonist self-administration curve are uncertain. The uncertainty makes the pharmacological analysis an interesting guessing game.

To shed more light on the potential dopamine independence of the reinforcing effects of PRE-084, its effects on dopamine concentrations in the nucleus accumbens shell of rats were examined (Garcés-Ramírez et al. 2011). The nucleus accumbens shell has been recognized as a critical brain structure for the effects of abused drugs (Pontieri et al. 1995; Tanda et al. 1997). PRE-084 produced a dose-related increase in extracellular dopamine at doses from 1.0 to 10 mg/kg, though the increase was substantially less than the maximal increase produced by cocaine (Fig. 4a). The absence of effects of PRE-084 in calcium-free Ringer's solution (Fig. 4b) indicated that the obtained increases in dopamine were the result of a physiological vesicular calcium-dependent release. Nonetheless, the effects of PRE-084 were significant only at the highest dose, which is 100-fold higher than the minimal self-administered dose (see filled symbols in Fig. 3e–h). This comparison of doses suggests that dopamine was not involved in the reinforcing effects of the lower self-administered doses of PRE-084. To further assess these effects of PRE-084 on dopamine, the effects of the σR antagonists, BD1063 and BD1008 were studied in combination with PRE-084 (Fig. 4c, d). In contrast to the self-administration results, neither antagonist appreciably altered the effects of PRE-084 on dopamine, consistent with the suggestion that reinforcing effects of PRE-084 were dopamine independent.

The results of these studies indicate that experience with indirect-acting dopamine agonists induces reinforcing effects of previously inactive σ_1R agonists. Because ongoing high rates of food reinforced behavior did not function similarly, and because changing the consequences of responses on two levers accordingly

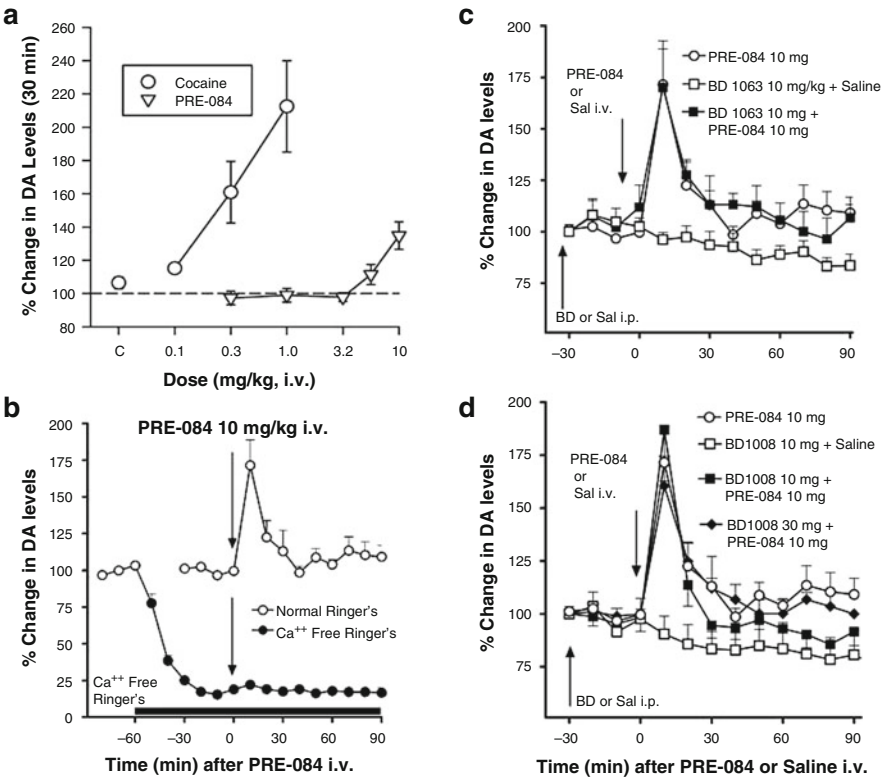

Fig. 4 Effects of an intravenous (i.v.) injection of PRE-084 on extracellular dopamine levels in the nucleus accumbens shell in rats. (**a**) Effects of a single i.v. injection of cocaine or PRE-084. Ordinates: Percent change in dopamine levels (for first 30 min after injection). Abscissae: Drug dose in mg/kg, log scale. Each point represents the mean (±SEM) of the amount of dopamine in dialysate samples, expressed as percentage of basal values ($N = 4$–8). Note that PRE-084 was more than 30-fold less potent than cocaine in increasing dopamine levels and less effective at the highest dose tested. (**b**) Effects of removal of calcium (Ca^{++}) from the perfusion Ringer's solution on PRE-084 induced increase in dopamine levels. Ordinates: Percent changes in dopamine levels at each 10-min sampling period. Abscissae: Time in min. Dopamine samples were collected each 10 min. Microdialysis probes were perfused with Ca^{++}-free Ringer's solution from 60 min before an injection of PRE-084 (*filled symbols above the solid line*). Each point represents the mean (±SEM) of dopamine levels expressed as percentage of basal values ($N = 4$). Note that PRE-084 was inactive when Ca^{++} was removed. (**c**, **d**) Effects of pretreatment with BD1063 (10 mg/kg i.p., a preferential σ_1R antagonist, or BD1008 (10–30 mg/kg i.p.), a nonselective σR antagonist, on PRE-084 induced increases in dopamine levels. Ordinates: Percent changes in dopamine levels at each 10-min sampling period. Abscissae: Time in min. Dopamine samples were collected every 10 min. BD1063 and BD1008 were administered 30 min before an injection of PRE-084 ($N = 4$). Note that the effects of PRE-084 were insensitive to the pretreatment with σR antagonists. Adapted from Garcés-Ramírez et al. (2011)

changed the behavior, this effect of the organism's history is not simply due to some kind of response persistence. The induction of σ_1R agonist self-administration, at this point, is related to the dopamine transporter as that target is common to cocaine and methamphetamine, but not heroin or ketamine. However, once induced the reinforcing effects of σ_1R agonists are independent of dopamine systems.

As indicated above, cocaine binds to σRs (Garcés-Ramírez et al. 2011; Sharkey et al. 1988), though with affinity less than that for the dopamine transporter (Garcés-Ramírez et al. 2011). However, a recent study with mice (Lever et al. 2016) indicates that in vivo doses of cocaine occupying σ_1Rs are about 2.5-fold higher than those that occupy the dopamine transporter. Further concentrations of cocaine in rat brain achieved with systemic injection are in the μM range (Nicolaysen et al. 1988; Pettit and Pettit 1994) and sufficient for binding to σ_1Rs. Affinity for σRs has also been reported for methamphetamine (e.g., Hiranita et al. 2014; Nguyen et al. 2005). Thus it is possible that actions at σ_1Rs contribute to the behavioral effects of cocaine involved in its abuse. It is further suggested that once induced, σ_1R agonist actions of these stimulants may function as an additional pathway by which these compounds exert their reinforcing effects. It is therefore hypothetically possible that this redundant pathway to reinforcement by these two stimulants may play an essential role in the intractability to medical treatment of stimulant abuse, particularly when those treatments target dopamine systems. This consideration suggests novel approaches for the development of combination chemotherapies to combat stimulant dependence.

σR Antagonist Effects on Stimulant Self-Administration As mentioned above, the first studies of interactions between σR agonists and stimulants found antagonism of locomotor stimulant effects and several subsequent studies documented that σR antagonists blocked place conditioning produced by various drugs of abuse (Table 5). However, the first attempt to block self-administration of a fixed unit dose of cocaine (0.25 mg/inj) with the σR antagonist, BD1047 failed to find an effect across a range of doses from 1 to 30 mg/kg (Martin-Fardon et al. 2007). Subsequent studies also reported a lack of effect on cocaine self-administration of several σR antagonists (BD1047, BD1008, BD1063, AC 927, NE100) across a wide range of antagonist doses as well as cocaine unit doses (Hiranita et al. 2010, 2011a).

Interestingly, the σR antagonist rimcazole and two of its analogs differed from other σR antagonists. Rimcazole was developed for the treatment of schizophrenia (Gilmore et al. 2004). However, the compound failed in clinical trials due to lack of efficacy as well as some frequency of seizures. Previous reports had suggested mixed results of interactions of rimcazole or its analogs with cocaine. For example, Matsumoto et al. (2001) showed antagonism of cocaine-induced acute toxicity (convulsions and lethality) by rimcazole and several of its analogs. In contrast, Katz et al. (2003) reported antagonism of locomotor stimulant effects; however, there were minimal effects if any of these compounds on cocaine discriminative-stimulus effects. In studies of cocaine self-administration (Hiranita et al. 2011a),

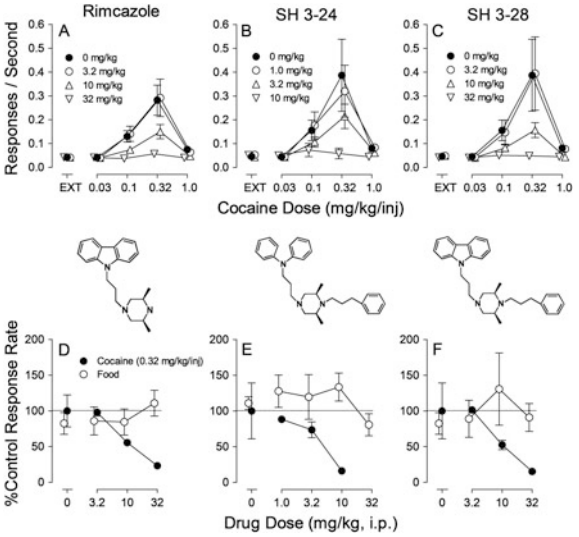

Fig. 5 Effects of presession treatments with rimcazole and its analogs (SH 3-24 and SH 3-28) on responding maintained by cocaine injections or food presentations. Each point represents the mean ± SEM ($N = 6$). Rimcazole and its analogs were administered i.p. at 5 min before sessions. (**A**–**C**) Effects of presession treatments with rimcazole and its analogs on cocaine self-administration. Ordinates: Responses per second. Abscissae: Cocaine injection dose in mg/kg, log scale. Note that rimcazole and its analogs dose-dependently decreased the maximal rates of responding maintained by cocaine injections. (**D**–**F**) Effects of presession treatments with rimcazole and its analogs on responding maintained by cocaine injections (0.32 mg/kg/injection) or food presentations (fourth component, each). Ordinates, response rates as percentage of control response rates (sessions before drug tests) for cocaine- and food-maintained responding. Abscissae, Drug dose in mg/kg (i.p.), log scale. A separate group of subjects were trained to respond to food reinforcement under an FR five-response schedule of reinforcement identical to that of cocaine self-administration. Rates of responding were from the fourth 20-min component of the session with responding maintained by injections of cocaine (0.32 mg/kg/injection) or food presentation. Note that rimcazole and its analogs were at least three-fold more potent in decreasing cocaine self-administration than in decreasing food-reinforced behavior. Adapted from Hiranita et al. (2011a)

rimcazole and its *N*-propylphenyl analogs, [3-(*cis*-3,5-dimethyl-4-[3-phenylpropyl]-1-piperazinyl)-propyl]diphenylamine hydrochloride (SH 3-24) and 9-[3-(*cis*-3,5-dimethyl-4-[3-phenylpropyl]-1-piperazinyl)-propyl]carbazole hydrobromide (SH 3-28), dose-dependently decreased maximal cocaine self-administration (Fig. 5, top panels). Additionally, these decreases in self-administration were selective, as they were obtained at doses that had no effects on comparable responding maintained by food reinforcement (Fig. 5, bottom panels).

Previous studies (Izenwasser et al. 1993; Valchar and Hanbauer 1993) indicated that rimcazole has affinity for the dopamine transporter as well as σRs. Subsequent studies confirmed that the analogs examined also had affinity for both the dopamine transporter and σRs (Hiranita et al. 2011a; Husbands et al. 1999; see Table 1).

Nonetheless, the combination of those actions seems an unlikely indication of targets that could produce the cocaine-antagonist effects found with rimcazole and its analogs. As indicated above selective σR antagonists have no effect on stimulant self-administration, whereas dopamine transport inhibitors enhance the self-administration of cocaine (Barrett et al. 2004; Hiranita et al. 2009; Schenk 2002). It was unclear on the face of it how a combination of inactivity and enhancement would produce a dose-dependent decrease in maximal cocaine self-administration.

To further examine this combination of effects selective σR antagonists and dopamine transport inhibitors were examined alone and in combination on cocaine self-administration in rats (Hiranita et al. 2011a). As is typical, the cocaine dose-effect curve was an inverted U-shaped function of cocaine unit dose per injection (Fig. 6A–C, filled symbols). A dose of nomifensine (0.32 mg/kg) that had no effect on cocaine self-administration when administered alone (data not shown) was studied in combination with the σR antagonists, BD1008, BD1047, and BD1063. The dose of nomifensine was the highest dose that did not shift the cocaine dose-effect curve leftward. As previously reported none of the σR antagonists had appreciable effects on cocaine self-administration. When administered in combination however there was a σR antagonist dose-dependent decrease in the maximal self-administration of cocaine (Fig. 6A–C, open symbols). Similar results were obtained with combinations of the dopamine uptake inhibitors, WIN 35,428 and methylphenidate, with these same σR antagonists (Hiranita et al. 2011a). Additionally, the combination of WIN 35,428 and BD1008 was found effective in blocking *d*-methamphetamine but not heroin or ketamine self-administration (Hiranita et al. 2014).

The combinations of σR antagonists and dopamine uptake inhibitors, as with rimcazole and its analogs, produced selective effects on cocaine self-administration (Hiranita et al. 2011a). Figure 6D–F shows that the particular combinations of nomifensine and σR antagonists that decreased cocaine self-administration produced little if any decrease in rates of responding maintained by food presentation in rats. That selectivity was also obtained with combinations of WIN 35,428 or methylphenidate and each of the σR antagonists, though the selectivity was somewhat reduced for the combination of WIN 35,428 and BD1063 (Hiranita et al. 2011a).

That similar interactions were obtained with structurally different dopamine uptake inhibitors and different σR antagonists suggests that this decrease in efficacy of cocaine in the self-administration procedure was not an idiosyncratic effect of a particular combination of compounds but was more generally a result of a fundamental dynamic interaction among the compounds. The somewhat different effects of WIN 35,428 in combination with BD1063 may be related to its particular pharmacology. WIN 35,428 has equal affinity for σ_1 and σ_2 receptor subtypes whereas the other two dopamine uptake inhibitors had higher affinity for σ_1 compared to σ_2 receptors (Hiranita et al. 2011a). BD1063 has greater selectivity for σ_1Rs compared to the other σR antagonists (Matsumoto et al. 1995; Table 1). Thus it is possible that the σ_2R affinity of WIN 35,428 is not sufficiently blocked by

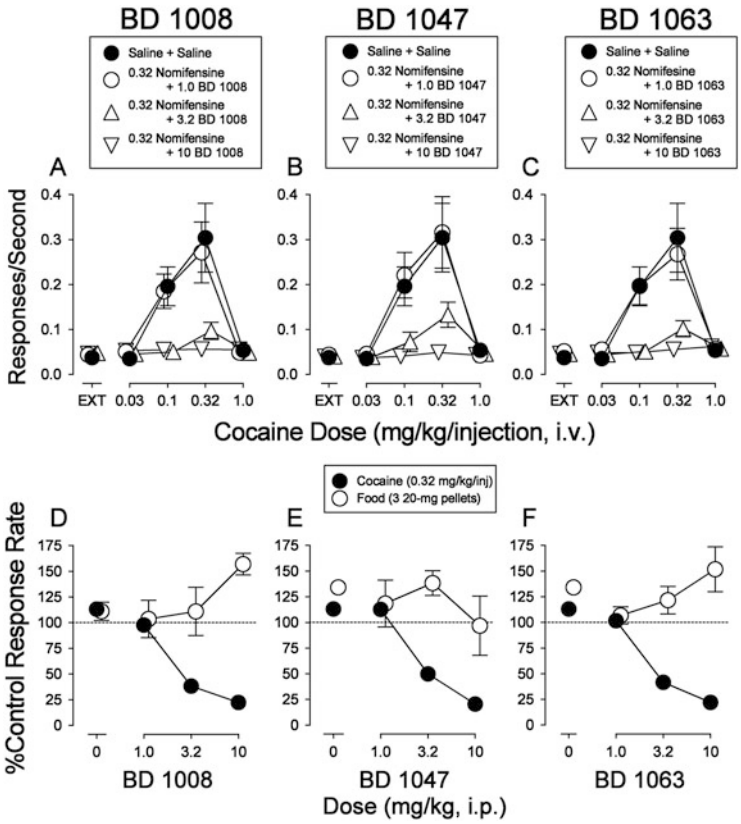

Fig. 6 Effects of presession treatments with dopamine uptake inhibitors combined with σR antagonists on cocaine self-administration. Ordinates: Responses per sec (**A**–**C**) or response rates as percentage of control response rates (sessions before drug tests) for cocaine- and food-maintained responding (**D**–**F**). Abscissae: Cocaine injection dose in mg/kg, log scale (**A**–**C**) or drug dose in mg/kg (i.p.), log scale (**D**–**F**). Each point represents the mean ± SEM ($N = 6$). Nomifensine (0.32 mg/kg, 5 min prior) was combined with BD1008, BD1047, and BD1063 (each at doses of 1.0, 3.2, and 10 mg/kg and at 5, 15, or 5 min prior to sessions, respectively). All injections were i.p. See Fig. 5 for more details. Note that each σR antagonist dose-dependently decreased the maximal rates of responding maintained by cocaine injections when combined with nomifensine. Also, the antagonism of cocaine self-administration was obtained at the combined doses that were inactive against food-reinforced responding. Adapted from Hiranita et al. (2011a)

BD1063. Speculating further, it is possible that a direct effect of WIN 35,428 on σ_2Rs interferes with the antagonism.

The discovery of highly selective σR subtype antagonists (Chu et al. 2015; James et al. 2012) allowed studies to address whether the antagonism of cocaine self-administration in rats by dual inhibition of the dopamine transporter and σRs was specific to a particular σR subtype (Katz et al. 2016). Radioligand displacement studies confirmed that CM 304 was approximately 600-fold selective for the σ_1R whereas CM 398 was 330-fold selective for the σ_2R subtype (Table 1; Katz et al.

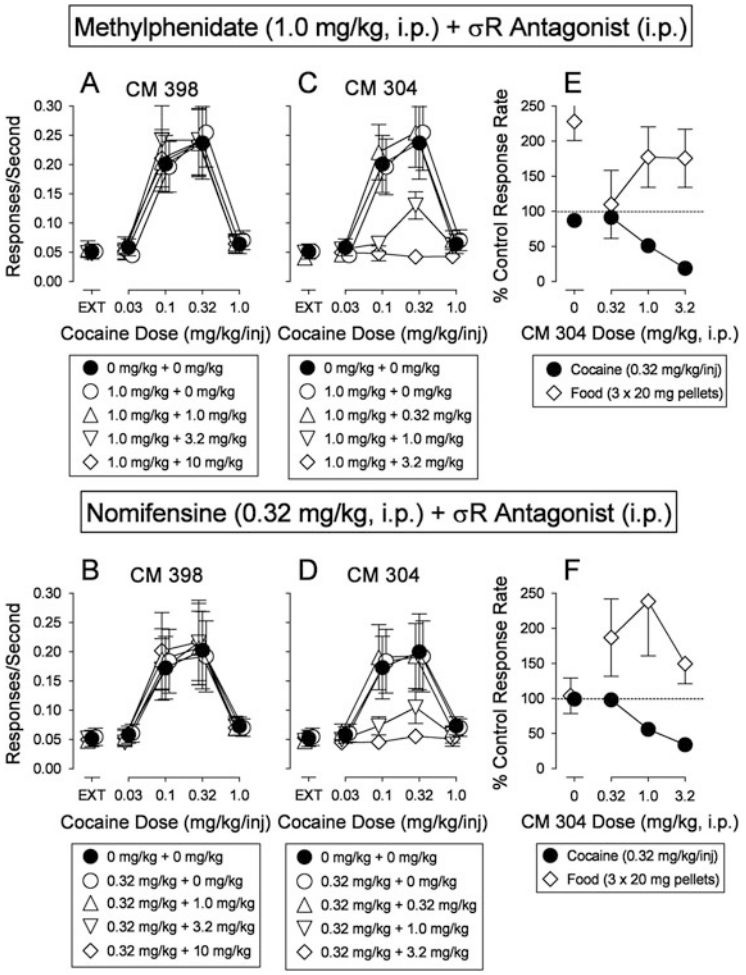

Fig. 7 Effects of presession treatments with dopamine uptake inhibitors (methylphenidate and nomifensine) combined with the selective σ_1 or σ_2 receptor antagonists, respectively, CM 304 or CM 398, on responding maintained by cocaine injections or food presentations. Each point represents the mean ± SEM ($N = 6$). All compounds were administered 5 min before sessions. (**A**–**D**) Ordinates: Response rates in responses per second. Abscissae: Cocaine injection dose (mg/kg, i.v.). Note that only CM 304 dose-dependently decreased the maximal rates of responding maintained by cocaine injections when combined with the dopamine uptake inhibitors. (**E**, **F**) Ordinates: Response rates as percentage of control response rates (sessions prior to drug tests). Abscissae: Dose in mg/kg of CM 304 administered i.p., log scale. See Fig. 5 for more details. Note that the antagonism of cocaine self-administration was obtained at the combined doses that were inactive against food-reinforced responding. Adapted from Katz et al. (2016)

2016). Combinations of CM 398 with either methylphenidate (1.0 mg/kg) or nomifensine (0.32 mg/kg) were without effects on cocaine self-administration (Fig. 7A, B). In contrast, CM 304 dose-dependently decreased maximal cocaine

self-administration when combined with either methylphenidate or nomifensine (Fig. 7C, D). Further, neither CM 304 nor CM 398 when administered alone was active in blocking cocaine self-administration. Most important, combinations of CM 304 with either dopamine uptake inhibitor decreased cocaine self-administration at dose combinations that had no effects on food-maintained responding (Fig. 7E, F). These results indicate that the selective effect of concomitant inhibition of dopamine transport and antagonism of σRs is due to specific blockade of σ_1Rs.

Reinstatement Martin-Fardon et al. (2007) examined the effects of BD1047 on "reinstatement" of cocaine self-administration. Though there are several variants of procedures so labeled, most involve three phases. In the first two of these, subjects are trained to self-administer a drug, followed by an extinction phase in which responses no longer produce injections (in some variants of the procedure this extinction phase is eliminated). In the final testing phase, various treatments are assessed for their effectiveness in increasing (reinstating) the now low rate of responding. The treatments that increase rates of responding during the reinstatement tests include response-independent administration of the drug previously self-administered immediately before the reinstatement test, subjecting the subjects to any of several kinds of noxious stimuli (e.g., electric shock) as presumed stressors, or presentation of stimuli previously paired with drug injections. It is typically asserted that an increase in the rate of responding during the reinstatement test is a model of relapse to drug taking, or that the renewed responding reflects craving for the drug. Various test drugs have been examined for their efficacy in blunting the reinstating effects of any of the above-mentioned treatments with the supposition that such a blunting is evidence of the potential for a therapeutic effect of the test drug. For a critical review of this procedure and its validation see Katz and Higgins (2003).

In the study by Martin-Fardon et al. (2007) responding was initially maintained by cocaine during sessions in which white noise was presented. During the extinction phase the white noise was turned off and cocaine injections were discontinued. When response rates decreased to low values, tests were conducted in which the white noise was again presented though cocaine injections remained unavailable. The reintroduction of white noise significantly increased rates of responding and that effect was attenuated by BD1047 at doses of 20 and 30 mg/kg. Additionally, a 1.5-fold higher dose was necessary to decrease reinstated responding in a separate group of subjects for which responding was formerly maintained by sweetened condensed milk, suggesting some selectivity of BD1047 for responding formerly maintained by cocaine. The authors note several cautions with regard to over-interpreting the obtained effects, in particular, that only one σR antagonist was examined (Martin-Fardon et al. 2007).

Results of a study on the effects of σR antagonists on self-administration of cocaine (Hiranita et al. 2010) may clarify the generality of the effects of σR antagonists on reinstatement phenomena (Martin-Fardon et al. 2007). In that study, i.v. cocaine was self-administered by rats during daily experimental sessions

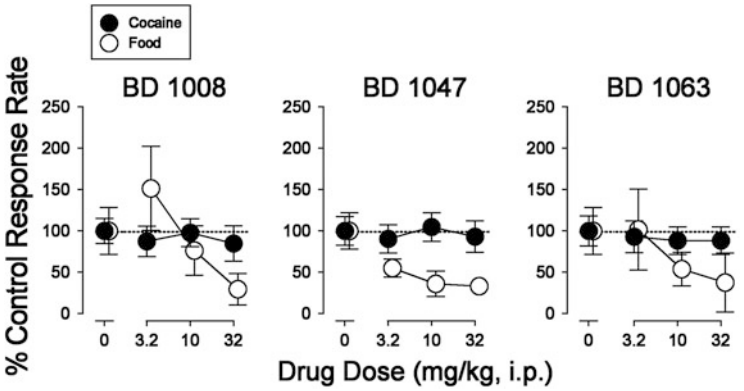

Fig. 8 Effects of presession treatments with selective σR antagonists on responding during the extinction component for cocaine injections or food presentations. Ordinates: Responses rates as percentage of control response rates (sessions before drug tests) during the extinction component. Abscissae: Drug dose in mg/kg (i.p.), log scale. Each point represents the mean ± SEM ($N = 6$). BD1008, BD1047, and BD1063 were administered i.p. at 5, 15, or 5 min prior to sessions, respectively. See Fig. 5 for more details. None of the tested σR antagonists had significant effects on responding during cocaine extinction. Further, the doses tested ranged from those having no effects to those that decreased responding during food extinction. Adapted from Hiranita et al. (2010)

that were divided into five components. Cocaine dose was systematically increased during the components following the first. In that initial component, as in many reinstatement procedures, responses produced only a change in the stimuli previously presented when cocaine was injected. A second group of rats was studied in an analogous manner but with food presentation rather than cocaine (Hiranita et al. 2010). Figure 8 shows rates of responding during that extinction component during sessions preceded by vehicle injections (points above 0) and those after treatment with the several σR antagonists (points above dose values of 3.2–32.0 mg/kg). None of the tested σR antagonists had significant effects on responding during cocaine extinction. Further, the doses tested ranged from those having no effects to those that decreased responding during food extinction. These findings indicate that selective decreases in responding during extinction or cocaine self-administration produced by σR antagonists on responding are limited in their generality.

Comment on Discrepancies in Results Obtained with Self-Administration and Place-Conditioning Procedures It is noteworthy that selective σR antagonists interfere with place conditioning induced by cocaine, but not its self-administration. These differences may be related to the species studied, as most of the place-conditioning studies, with the exception of Mori et al. (2014a), used mice. In contrast, the self-administration studies were conducted using rats. The difference may also be due to differences in the fundamental nature of the assessments of "reinforcing" effects inherent in the two procedures. In place conditioning there

is a contingency relation between two stimuli, whereas in self-administration procedures there is a contingency relation between a response and a stimulus.

As originally noted by Martin-Fardon et al. (2007), the studies showing a lack of effect of σR antagonists on cocaine self-administration were with behavior that was previously established whereas the place-conditioning studies assessed the effects of σR antagonists on the acquisition of the conditioned behavior. It would therefore be of value to assess the effects of σR antagonists on the acquisition of cocaine self-administration. Extensive studies by T. Maurice and colleagues have shown various σR agonists can reverse learning and memory deficits in a variety of preclinical models (for a review see Maurice 2007), suggesting that those effects may underlie the effects of σR antagonists on place conditioning. However, in most studies on learning and memory σR antagonists have been inactive when assessed alone. However, as those assessments have most often been controls for studies of interactions with σR agonists, it remains possible that broader studies might reveal actions in procedures designed to assess learning and memory.

If indeed the effects of σR antagonists on stimulant place conditioning were due to a more generalized impairment of conditioning, the effect should be evident in place-conditioning procedures independently of the drug used as an unconditional stimulus. Indeed, there are several studies showing blockade by various σR antagonists of place conditioning induced by compounds from several pharmacological classes, including ethanol (Bhutada et al. 2012; Maurice et al. 2003), morphine (Chen et al. 2011; Vidal-Torres et al. 2013; Wu et al. 2007), and U-50,488H (Mori et al. 2012). Complicating the picture are a few reports of attenuation of place conditioning by the σR agonist SA4503 (Horan et al. 2001; Mori et al. 2012). Nonetheless, these studies suggest that the interference of place conditioning by σR antagonists may not be specific to stimulants and may occur more generally, an outcome consistent with an effect on the conditioning process rather than the specific reinforcing effects of the stimulants.

Whatever the ultimate behavioral mechanism of the effects of σR antagonists on place conditioning, the seemingly disparate outcome compared to the results with stimulant self-administration procedures emphasizes that a consideration of the methods used, and the environmental circumstances surrounding the effects, can be critical to the outcome. The behaviors expressed in self-administration, place conditioning, and other procedures used to assess reinforcing effects are a function of a limited set of overlapping variables. Behavior is complexly determined, and as such, drugs may have quite different effects that depend on the conditions of the study. Stated differently, it would be foolish to consider these different behavioral procedures as simply a “read out” of a unitary underlying neuronal circuit.

5 Working Hypotheses for Mechanisms Underlying σR: Dopamine Transporter Interactions

The induction of σR agonist self-administration by stimulants together with the data on the antagonism of cocaine self-administration by combinations of σR antagonists and dopamine transport inhibitors suggests a mechanism that involves both short-term and long-term effects. The induction of reinforcing effects of σR agonists by stimulant self-administration likely relies upon an immediate interaction of the σR and the dopamine transporter. Preliminary studies by Khoshbouei and colleagues (Lin et al. 2012) and by Hong et al. (2013) have documented such interactions. These studies have shown the co-immunoprecipitation of σ_1R and dopamine transporter in transfected cells, suggesting potential direct protein–protein interactions among these entities. This interaction appears to involve the transmembrane domain of σ_1R, as a splice variant of σ_1R lacking most of the C-terminal domain showed a stronger association with the dopamine transporter, compared with the full-length σ_1R (Hong et al. 2013). Interestingly co-immunoprecipitation was enhanced in the presence of methamphetamine (Lin et al. 2012).

It has been reported that increased membrane cholesterol content enhanced binding B_{max} of the cocaine analogue, WIN 35,428, to the dopamine transporter. Further, substituted cysteine accessibility studies suggested that the effect of cholesterol was due to a change in the conformational equilibrium of the dopamine transporter to favor a conformation open to the extracellular space (Hong and Amara 2010). Recent crystal structures of the *Drosophila* dopamine transporter (Penmatsa et al. 2013; Wang et al. 2015) showed the association of cholesterol with the dopamine transporter protein. Further, it was suggested that cholesterol may hinder the movement of transmembrane segment 1a of the dopamine transporter and stabilize an open-to-out conformation (Penmatsa et al. 2013). Thus, cholesterol may play a role in regulating the function of the dopamine transporter.

Palmer et al. (2007) previously indicated that cholesterol binds to σ_1Rs, and Ruoho and colleagues (Pal et al. 2007, 2008; Fontanilla et al. 2008) identified putative steroid-binding-like domains I and II on σ_1Rs. It is tempting to speculate that in membrane domains where σ_1R and the dopamine transporter form intricate interactions, binding of cholesterol to σ_1Rs may regulate the availability of cholesterol to the dopamine transporter, thereby shifting the equilibrium of transporter proteins between outward-facing and inward-facing conformations. Other studies have suggested that compounds that prefer binding to the inward-facing state of dopamine transporter have diminished stimulant-like actions and may antagonize the effects of standard stimulants (see review by Reith et al. 2015). The recent elucidation of X-ray crystal structure of human σ_1R represents an exciting breakthrough (Schmidt et al. 2016). Different from the two transmembrane-domain topology model derived from previous biochemical studies, the structure shows a homo-trimer of σ_1R, with each protomer exhibiting a single transmembrane domain and a cytoplasmic domain containing a ligand-binding pocket. As this structure presents a static snapshot of σ_1R, it will be fascinating to explore how binding of

σ_1R agonists, antagonists, and potentially cholesterol or other membrane lipids to σ_1R dynamically modulates its conformation and differentially affects the interaction of σ_1R with its protein partners.

The behavioral pharmacology of σ_1R agonist self-administration suggests a long-term change in which dopamine systems are uninvolved. The pharmacology of σ_1R agonist self-administration is different from common drugs of abuse. The chaperone functionality of the σ_1R protein (Hayashi and Su 2007) suggests that it may be having its effects by interacting with another protein, and the induction of reinforcing effects of σ_1R agonists virtually ensures that the effect involves a partner membrane protein. As detailed above, the elucidation of molecular mechanisms by which σ_1Rs regulate plasma membrane events is expanding. The involvement of σ_1Rs with several other proteins, including mu- and delta-opioid receptors (Kim et al. 2010), potassium Kv1.2 channels (Kourrich et al. 2013), as well as σ_1-dopamine D_1 (Navarro et al. 2010) or D_2 (Navarro et al. 2013) receptor heteromers has been studied. However, these findings should not induce tunnel vision excluding other candidate σ_1R partner proteins. The mechanisms underlying long-term changes remain unclear and may involve more than one particular protein partner.

In summary, it is clear that actions of stimulants related to their abuse induce unique changes in σ_1R activity. Further, these potential changes create redundant, and once established independent, reinforcement pathways. These redundant pathways may contribute to the treatment-resistant nature of stimulant abuse. Further, concomitant targeting of both the well-known reinforcing pathways initiated by blockade of the dopamine transporter, as well as σ_1R proteins produces an effective and selective antagonism of stimulant self-administration, suggesting new avenues for combination chemotherapies to specifically combat stimulant abuse.

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Subjects used in the studies published from the NIDA Intramural Research Program were maintained in facilities fully accredited by the American Association for the Accreditation of Laboratory Animal Care, and those experiments were conducted in accordance with the guidelines of the institute's Animal Care and Use Committee.

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Sigma Receptors and Alcohol Use Disorders

Valentina Sabino and Pietro Cottone

Contents

Abstract

Although extensive research has focused on understanding the neurobiological mechanisms underlying alcohol addiction, pharmacological treatments for alcohol use disorders are very limited and not always effective. This constraint has encouraged the search for novel pharmacological targets for alcoholism therapy. Sigma receptors were shown to mediate some of the properties of cocaine and amphetamine, which was attributed to the direct binding of psychostimulants to these receptors. More recently, the role of sigma receptors in the rewarding and

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reinforcing effects of alcohol was also proposed, and it was suggested that their hyperactivity may result in excessive alcohol drinking. This chapter reviews current knowledge on the topic, and suggests that the sigma receptor system may represent a new therapeutic target for the treatment of alcohol use disorders.

Keywords
Addiction • Alcohol • Alcoholism • Alcohol dependence • Consumption • Drinking • Ethanol • Preferring • Withdrawal

1 Epidemiology and Associated Medical Conditions

The global status report on alcohol and health by the World Health Organization (WHO) indicates that worldwide alcohol consumption in 2010 was equal to 6.2 l of pure alcohol consumed per person aged 15 years or older per day (World Health Organization 2014). The WHO also indicates that in 2012, over 3 million deaths (~6% of all global deaths) were attributable to alcohol consumption (World Health Organization 2014). Globally, alcohol misuse is the first risk factor for premature death and disability for people between the ages of 15 and 49, and it is ranked fifth when all ages are accounted for. One-fourth of total deaths in people between 20 and 39 years are dependent on alcohol (World Health Organization 2014; Lim et al. 2012). In addition, estimates of the global economic burden of alcohol consumption suggest that alcohol is responsible for 1.3–3.3% of total health costs, 6.4–14.4% of total public order and safety costs, 0.3–1.4% of gross domestic product GDP for criminal damage costs, 1.0–1.7% of GDP for drunk driving costs, and 2.7–10.9% of GDP for workplace costs (e.g., absenteeism, unemployment, and premature mortality) (Baumberg 2006).

The adverse consequences on health associated with alcohol consumption are numerous. The WHO indicates that alcohol is a causal factor in 60 types of diseases and injury-related health conditions, including addiction, gastrointestinal diseases, cardiovascular diseases, cancers, fetal alcohol spectrum disease, and alcohol-related injuries (World Health Organization 2014).

Acute alcohol consumption is responsible for a variety of physiological and behavioral effects which are resultant of blood alcohol concentrations (BACs), (Koob and Le Moal 2005). At BACs of 10–50 mg/dl, alcohol increases locomotor activity, disinhibits behavior, and relieves anxiety. When BACs reach 80 mg/dl, alcohol impairs judgment, cognition, and motor function. Individuals with BACs of 150 mg/dl experience marked motor impairment and ataxia, memory lapse, as well as decreased reaction time. BACs of 300 mg/dl produce hypnosis and can cause general anesthesia and coma. At BACs of 400 mg/dl, death is observed in 50% of the people (Koob and Le Moal 2005).

Alcohol is responsible for a plethora of psychiatric disorders, the most relevant being alcohol use disorder (AUD). The diagnosis of AUD in the Diagnostic and

Statistical Manual of Mental Disorders, Fifth Edition (DSM-5, American Psychiatric Association 2013) integrates in a single disorder the diagnoses of alcohol abuse and alcohol dependence previously described in the fourth edition of the manual. The manual lists 11 criteria for AUD, and the disorder is diagnosed as mild, moderate, or severe as a function of the number of criteria met (2–3 mild, 4–5 moderate, >5 severe). According to the DSM-5, the diagnosis of AUD is therefore based on the presence of impaired control, social impairment, risky use, as well as pharmacological indicators.

2 Definitions of Alcohol Use Disorders

Alcohol represents the most commonly used and abused substance in the world and it has been consumed for centuries in several cultures. Alcohol exerts beneficial effects when consumed in moderation, but it has abuse potential when consumed in excess. According to the Dietary Guidelines for Americans, moderate alcohol consumption is defined as up to one drink per day for women and up to two drinks per day for men. A standard drink is defined as 14 g of pure alcohol, which are equivalent to a 12-ounce can of beer, a 5-ounce glass of wine, or a 1.5-ounce glass of 80-proof liquor. The National Institute on Alcohol Abuse and Alcoholism (NIAAA) defines binge drinking as a pattern of drinking which results in BAC levels of 80 mg/dL (NIAAA 2004). Binge drinking typically occurs with four drinks for women and five drinks for men in a time window of approximately 2 h. The Substance Abuse and Mental Health Services Administration (SAMHSA) defines binge drinking as drinking five or more alcoholic drinks on the same occasion on at least 1 day in the past 30 days, while heavy drinking is defined as drinking five or more drinks on the same occasion on each of 5 or more days in the past 30 days (Koob and Le Moal 2005).

3 Molecular Targets of Alcohol

The molecular mechanisms of action of alcohol are several and complex, and still not entirely understood. The complexity of alcohol mechanisms is mainly due to its molecular structure: alcohol is a very small molecule with both polar and nonpolar properties and as such it can easily travel through both hydrophilic and lipophilic molecular and cellular structures. As a consequence, alcohol interacts with both plasma membrane and intracellular proteins. Given the plethora of molecular effects that alcohol can produce, here we will limit our discussion to a brief description of the main mechanisms underlying ethanol's putative direct interaction with specific target proteins.

A well-known mechanism of action of alcohol is related to its direct interaction with ligand-gated ion channel membrane proteins, especially the pentameric (five

subunits) Cys-loop superfamily of neurotransmitter receptors including GABAA receptor (GABAAR), nicotinic acetylcholine receptor (nAChRs), and glycine receptor (GlyR) (Olsen et al. 2014; Trudell et al. 2014). Alcohol directly binds and agonizes GABAAR, and the specific receptor subunit composition makes it more or less responsive to ethanol (Lobo and Harris 2008; Santhakumar et al. 2007; Mehta and Ticku 1988; Suzdak et al. 1988). α4β2δ, α4β3δ, and α6β3δ GABAARs are very sensitive to alcohol, with concentrations of 0.1–1 mM of ethanol significantly enhancing GABA currents (Sundstrom-Poromaa et al. 2002; Wallner et al. 2003). In addition, alcohol is hypothesized to directly act on nAChRs and the net effect of this interaction depends on the receptor subunit composition; alcohol enhances the function of α4β2, α4β4, α2β2, and α2β4 nAChRs, while it exerts no effect on α3β2 and α3β4 nAChRs, and inhibits α7 nAChRs (Narahashi et al. 1999; Cardoso et al. 1999; Davis and de Fiebre 2006). Furthermore, alcohol can bind and positively modulate GlyRs (Perkins et al. 2010).

Another well-described mechanism of action of alcohol is its antagonistic action on the N-methyl-D-aspartate glutamate receptor (NMDAR); alcohol is thought to interact allosterically with NMDARs, reducing the affinity of the agonist for the receptors (Lima-Landman and Albuquerque 1989; Wright et al. 1996).

Alcohol has also been demonstrated to directly interact with G-protein-gated inwardly rectifying potassium (GIRK) channels activating them through a direct binding to a hydrophobic pocket. Interestingly, GIRK channels can be occupied and activated by chemical groups different than those of alcohol (Bodhinathan and Slesinger 2013).

Sigma receptor (SigR) ligands have been shown to influence the effects of psychostimulants, in particular cocaine and methamphetamine, which were demonstrated to bind directly to SigR, although at low (micromolar) affinity (Brammer et al. 2006; Nguyen et al. 2005; Sharkey et al. 1988). For this reason, until a few years ago, only a few studies had examined the possibility of a SigR modulation of ethanol's actions. However, growing evidence indicates that indirect SigR-mediated effects may exist for other substances of abuse besides psychostimulants, including ethanol. Therefore it is conceivable that, for example in the context of cocaine, some of the molecular mechanisms described for SigR may also be common to those of alcohol. Important mechanisms include the described interactions of SigR with dopamine D1 and D2 receptors, potassium channels and opioid receptors, as well as proteins of the nuclear envelope and histone deacetylases (Navarro et al. 2010, 2013; Kourrich et al. 2013; Tsai et al. 2015; Kim et al. 2010; Mei and Pasternak 2007).

4 Sigma Receptors and the Locomotor-Activating and Sedative Effects of Alcohol

Alcohol effects on locomotor activity are a direct function of the BACs attained. At low BACs, alcohol exerts locomotor-stimulating effects and increases locomotor activity, while at higher BACs, the depressant and sedative effects of alcohol become evident. The locomotor-stimulating properties of alcohol are interpreted as an

index of its rewarding properties and abuse liability, and they are thought to be dependent on the activation of the mesolimbic dopaminergic system (Phillips and Shen 1996). In rodents, the locomotor-stimulating effects of alcohol and drugs are typically evaluated by placing subjects in an arena equipped with infrared sensor photobeams; the interruption of these photobeams, caused by the subjects' movement, is recorded by a computer and the number of interruptions is a direct index of the locomotor activity of the subjects.

The selective sigma-1 receptor (Sig-1R) antagonist N-[2-(3,4-dichlorophenyl)ethyl]-N-methyl-2-(dimethylamino)ethylamine (BD1047), injected at doses of 3–30 mg/kg, dose-dependently blocked the locomotor-stimulating effects induced by 1 g/kg of ethanol in Swiss mice (Maurice et al. 2003). In the same study, it was shown that the selective Sig-1R agonist PRE-084, administered at doses of 1–10 mg/kg, failed to affect alcohol-induced locomotion stimulation. Interestingly, neither drug affected locomotor activity when administered alone (Maurice et al. 2003).

Accordingly, in a recent study, Valenza et al. (2015) found that C57BL/6J mice lacking the *SIGMAR1* (previously known as *Oprs1*) gene, which encodes the Sig-1R, were less sensitive to the locomotor-stimulant effects of 1.5 g/kg of ethanol as compared to the wild-type counterpart. Since the C57BL/6J strain is particularly insensitive to the locomotor-stimulant effects of ethanol, mice in this study were pretreated with the benzodiazepine (BDZ) partial inverse agonist Ro 15-4513 (Miczek and Weerts 1987), which is able to unmask the stimulant effects of ethanol by blocking the depressant properties of ethanol (Becker and Hale 1989). These observations, therefore, confirm the notion that Sig-1R is involved in mediating the locomotor-stimulating effects of alcohol. Together these studies suggest that Sig-1R activation may mediate or at least contribute to the locomotor-activating effects of ethanol, and therefore perhaps also to its abuse potential.

In the same study, the effects of *SIGMAR1* knockout (KO) on the sedative effects of high doses of alcohol were tested using the loss of righting reflex procedure. In this procedure, following the administration of a high dose of alcohol (4 g/kg), mice are placed on a V-shaped surface, and the latency to lose the righting reflex (inability to right itself from a supine position) and the sleep duration are recorded. *SIGMAR1* KO mice were shown not to differ from wild-type mice neither in the latency to lose the righting reflex nor in time spent sleeping, suggesting a similar sensitivity between the two genotypes and therefore opposing the involvement of Sig-1R in the sedative effects of alcohol (Valenza et al. 2015).

5 Sigma Receptors and the Rewarding Properties of Alcohol

Alcohol can increase the salience of the contextual stimuli, such as places in which positive alcohol effects are experienced. Once, through associative learning, contextual neutral stimuli have acquired rewarding properties, they can then exert powerful control over behavior. This mechanism plays a critical role in maintaining alcohol taking behavior, as approaching an alcohol-associated context can set the occasion for drinking to begin (Bardo and Bevins 2000). An experimental

procedure to evaluate whether a substance has rewarding properties is place conditioning (also known as conditioned place preference), a task where a compartment equipped with specific contextual cues is repeatedly paired with a rewarding substance (in this case ethanol) and therefore becomes preferred versus a second, neutral compartment (Bardo and Bevins 2000). Even though technically challenging depending on the specific species and strain used, alcohol is able to induce conditioned place preference in rodents (Cunningham and Noble 1992). Pharmacological agents can be administered either before each of the conditioning sessions to assess their influence on the acquisition of place preference or before the post-conditioning test to instead assess their influence on the expression of place preference.

Sig-1R antagonism has been shown to successfully block the expression of the conditioned place preference induced by alcohol. Indeed, pretreatment with the selective Sig-1R antagonist BD1047 (3–30 mg/kg), administered during conditioning, has been shown to dose dependently block the acquisition of place preference induced by repeated injections of 2 g/kg of alcohol in male mice (Maurice et al. 2003). In the same study, the authors demonstrated a bidirectionality of the process, as the selective Sig-1R agonist 2-(4-morpholino) ethyl 1-phenylcyclohexane-1-carboxylate (PRE-084, 1–10 mg/kg), given before a dose of ethanol (0.5 g/kg) (which was per se inert), resulted in a dramatic dose-dependent facilitation of ethanol-induced place preference (Maurice et al. 2003). These results were confirmed and extended in a study in which Sig-1R ligands were administered intracerebroventricularly (Bhutada et al. 2012). In this study, BD1047 (0.1–10 μg/mouse) dose dependently blocked not only the acquisition, but also the expression of ethanol-induced conditioned place preference. It is important to note that both BD1047 and PRE-084 have been repeatedly shown not to exert any effect on place preference when administered alone (Romieu et al. 2000, 2002; Maurice et al. 2003).

6 Sigma Receptors and Alcohol Drinking

Strong evidence from both human and animal studies supports the overarching hypothesis that SigR activation modulates alcohol intake and proposes a role for Sig-1R antagonists as potential pharmacological agents for the treatment of alcohol-use disorder.

A functional relationship between alcoholism and polymorphisms in the human *SIGMAR1* gene has been shown in a study by Miyatake et al. (2004), who measured the differential representation of *SIGMAR1* functional polymorphisms in a Japanese population of alcoholic subjects. This study showed that the frequency of the A-485 allele and the TT-241-240/Pro2 haplotype, whose transcriptional activity was significantly reduced compared with that of the T-485 allele and the GC-241-240 allele, was higher in controls relative to alcoholic subjects, suggesting that this polymorphism in *SIGMAR1* may act as protective factors for alcohol dependence.

At a preclinical level, a relatively large body of evidence has shown a bidirectional role for SigRs in regulating alcohol drinking, and these studies are reviewed

below based on the experimental procedure used to assess alcohol drinking behavior: home cage vs. operant self-administration.

6.1 Home Cage Drinking

A procedure used to evaluate drinking in rats is the two-bottle choice. In this procedure, alcohol drinking is measured in rats that are provided continuous access (24-h day) in their home cage with two bottles: one containing a solution of ethanol (usually 10% *v*/*v*), and the other one water. Intake and preference are both measured.

In the context of SigR pharmacology, many studies using the two-bottle choice procedure have been performed in selectively bred Sardinian alcohol-preferring (sP) rat. Lines of rodents genetically selected for high alcohol intake and preference represent a successful tool to study the genetic factors underlying excessive alcohol consumption (Ciccocioppo and Hyytia 2006). In particular, rats of the sP rat line have been shown to voluntarily drink large quantities of ethanol, to have a strong innate preference for ethanol over water, and to possess a heritable component analog to human alcohol dependence (Cloninger et al. 1981; Prescott and Kendler 1999; Sigvardsson et al. 1996). Therefore, sP rats represent a model of genetic predisposition to high ethanol drinking and a tool for identifying potential pharmacotherapies for alcoholism (Colombo et al. 2006).

Sig-1Rs have been demonstrated to exert a key role in both the acquisition and the maintenance of excessive alcohol drinking in sP rats. Sabino and colleagues have shown that chronic systemic administration of the selective Sig-1R antagonist BD1063 (30 mg/kg) dramatically reduced the acquisition of alcohol-drinking behavior in sP rats, reducing both intake and preference for alcohol (Blasio et al. 2015). In this study, vehicle-treated sP rats rapidly escalated their alcohol intake to 6 g/kg of ethanol per day within the 2 weeks of observation. Ethanol drinking acquisition was also accompanied by a rapid increase in the preference for alcohol as the consumption of water gradually decreased to maintain a stable overall fluid intake. On the other hand, BD1063-treated sP rats showed a marked reduction in alcohol drinking accompanied by an increase in water intake. Notably, the drug treatment did not affect overall fluid intake and significantly decreased the preference for alcohol, indicating that Sig-1R antagonism is able to shift the innate inclination to drink alcohol over water of sP rats (Blasio et al. 2015). sP rats were also shown to have innately higher levels of Sig-1R protein in the nucleus accumbens (NAcc) as compared to outbred Wistar rats, which provides critical information about the genetic basis of high alcohol drinking (Blasio et al. 2015). Interestingly, increased Sig-1R levels in the NAcc were normalized by chronic alcohol consumption, which may be consistent with the reduced motivation to drink alcohol which follows recent alcohol consumption (Blasio et al. 2015).

Sig-1R antagonism has also been demonstrated to decrease the maintenance of alcohol drinking in sP rats (Sabino et al. 2009b). The selective Sig-1R antagonist

NE100 (10–30 mg/kg) reduced the intake of alcohol consumed by sP rats when injected either acutely or chronically. Following acute administration, NE100 dramatically reduced excessive ethanol intake, and decreased the preference for alcohol by increasing the volume of water consumed without affecting total fluid intake. In addition, when sP rats were offered a two-bottle choice between sucrose and water, acute NE100 treatment did not decrease the consumption of sucrose. Overall, these results suggest that the effect of the drug was selective for alcohol and that it was not due to malaise or secondary to an overall behavioral deficit (Sabino et al. 2009b). In addition, the alcohol-suppressive effect of NE100 was not due to changes in ethanol pharmacokinetics, as drug treatment did not affect BACs when ethanol was administered by gavage (Sabino et al. 2009b). Chronic NE100 treatment to sP rats (30 mg/kg) also significantly reduced alcohol intake, with a peak reduction by the treatment day 3. Starting from day 6, some tolerance to NE100's effect was evident, similar to what was also observed with opioid receptor antagonists (e.g., naloxone and naltrexone), for which tolerance has been shown to develop after 5–14 days of treatment (Cowen et al. 1999; Overstreet et al. 1999; Parkes and Sinclair 2000). Chronic treatment with NE100 did not affect daily food intake (Sabino et al. 2009b).

NE100 treatment was also shown to fully block the increase in alcohol consumption observed when alcohol access is reinstated following a period of deprivation (Sabino et al. 2009b). This transient increase in alcohol consumption is referred to as "alcohol deprivation effect" and it has been posited to be an animal model for alcohol craving and relapse (Rodd-Henricks et al. 2000; Agabio et al. 2000). In this procedure, sP rats, trained under a two-bottle choice continuous access condition, were forced to abstain from alcohol for 1 week, and on the test day, either NE100 or vehicle was administered to the rats before access renewal. Under vehicle conditions, abstinent sP rats dramatically increased the intake of alcohol upon renewing access to the bottle of alcohol as compared to non-abstinent rats; this alcohol deprivation effect was fully prevented by pretreatment with the selective Sig-1R antagonist (Sabino et al. 2009b).

It has been recently shown that *SIGMAR1* KO mice show greater alcohol intake and greater alcohol preference in a two-bottle choice procedure as compared to WT mice (Valenza et al. 2015). Interestingly, the higher the concentration of alcohol provided (3%, 6%, and 20% *v*/*v*), the more pronounced the observed increase in alcohol intake was. Conversely, when mice were tested in two-bottle choice for either saccharin or quinine, neither the intake of the sweet nor of the bitter solution was changed in *SIGMAR1* KO mice, ruling out that the deletion of *SIGMAR1* results in altered taste perception or in a general increase in intake of fluids (Valenza et al. 2015). Results from this study seem to contradict the overarching hypothesis that Sig-1R activation mediates the effects of alcohol and that Sig-1R antagonism decreases excessive alcohol drinking (Sabino et al. 2009a, b, 2011). However, the species difference (mice vs. rats) may be responsible for the differential effects observed. In addition, it cannot be excluded that in whole-body KO mice developmental mechanisms play a counteradaptive role and may confound the results obtained.

6.2 Operant Self-Administration

The reinforcing effects of alcohol are studied using instrumental conditioning, a form of associative learning in which subjects (typically rats or mice) learn to self-administer alcohol (or water) by pressing a lever inside an operant chamber. Following a single press on one of the two levers (fixed ratio 1), a syringe pump containing the solution is activated and the respective fluid is dispensed into a drinking cup. In this procedure alcohol drinking is evaluated as the number of responses emitted on the alcohol lever.

Two major studies have been pivotal in demonstrating the bidirectional modulatory role of SigR in the reinforcing properties of alcohol.

In a first study, the effects of the selective Sig-1R antagonist BD1063 on alcohol reinforcement were evaluated in both a genetic and an environmental animal model of excessive alcohol drinking (Sabino et al. 2009a). The genetic animal model used in this study was the sP rats described above. The environmental animal model was outbred Wistar rats made dependent through the exposure to chronic intermittent ethanol (CIE). Briefly, rats were housed for a period of 4–6 weeks in sealed chambers into which ethanol vapor was intermittently introduced (for review, see Vendruscolo and Roberts 2014); BACs were kept at approximately 150–200 mg% across the exposure period. During acute withdrawal from alcohol, CIE rats show heightened levels of ethanol self-administration, anxiety-like behavior, and increased threshold in the intracranial self-stimulation, compared to control, air-exposed rats (Sabino et al. 2006; Funk et al. 2006; O'Dell et al. 2004). Results from this study showed that the selective Sig-1R antagonist BD1063 (3.3–11 mg/kg) dose dependently reduced excessive ethanol self-administration in both sP rats and CIE rats during acute withdrawal (Sabino et al. 2009a). BD1063 did not, however, reduce ethanol self-administration in control rats. In addition, BD1063 treatment did not affect responding for water or for an equally reinforcing solution of saccharin, suggesting that the Sig-1R antagonist effects were selective for alcohol (Sabino et al. 2009a).

In the same study, the effects of BD1063 were also tested in a progressive ratio schedule of reinforcement for alcohol, which represents a highly validated operant model to assess subjects' motivation for alcohol (Hodos 1961). In this procedure, the number of lever presses (ratio) required to obtain a single reinforcer increases progressively, with the last ratio defined as the "breakpoint." The breakpoint, therefore, represents the maximum effort a subject expends to obtain the desired reinforcing stimulus, and is an objective measure of the subject's motivation. Results from this study showed that BD1063 (3.3–11 mg/kg) dose dependently reduced the breakpoint for ethanol in sP rats (Sabino et al. 2009a).

Collectively, these data suggest that Sig-1Rs are recruited in conditions of excessive ethanol intake and/or heightened motivation, thus likely contributing to innate and ethanol-induced increases in susceptibility to drink excessively. In addition, the increase in the NAcc Sig-1R expression levels in sP rats compared to outbred Wistar rats observed by Blasio et al. (2015) can be speculated to explain the increased

sensitivity of sP rats to pharmacological blockade with Sig-1R antagonists found in this study.

The results of a second study demonstrated the bidirectionality of the modulation of ethanol drinking exerted by the SigR system. Daily systemic treatment (2/day for 7 consecutive days) with the SigR agonist 1,3-di-(2-tolyl)guanidine (DTG) (15 mg/kg) was shown to increase ethanol self-administration in sP rats under a fixed ratio 1 schedule of responding (Sabino et al. 2011). Importantly, the increased self-administration in DTG-treated rats resulted in BACs exceeding 80 mg%, which can therefore be regarded as "binge-like" according to the definition provided by the National Institute on Alcohol Abuse and Alcoholism (NIAAA 2004). Importantly, SigR agonist treatment might represent a novel way to induce binge drinking in laboratory animals, which historically has been difficult to achieve (Sabino et al. 2011). Treatment with DTG also increased breakpoint for ethanol in a progressive ratio schedule of reinforcement, suggesting a greater motivation to work for alcohol. Notably, the DTG-induced increase in ethanol intake was reversed by a subthreshold dose of the Sig-1R antagonist BD1063, confirming that the Sig-1R subtype mediated the DTG effects (Sabino et al. 2011). In addition, considering that both sP rats and acutely withdrawn CIE rats show alterations of Sig-1R levels in the NAcc (Blasio et al. 2015; Sabino et al. 2009a), it is conceivable that Sig-1R of the NAcc may mediate the susceptibility to excessive drinking, both innate and induced by chronic alcohol exposure.

Repeated treatment with DTG induced an increase in μ- and δ-opioid receptor gene expression in the ventral tegmental area (VTA) of sP rats, suggesting that SigR agonists may facilitate ethanol's ability to activate the mesolimbic dopaminergic system through this mechanism which involved the endogenous opioid system of the VTA. These results suggest a key facilitatory role for SigR in the reinforcing effects of ethanol and identify a potential mechanism that contributes to excessive drinking.

7 Sigma Receptors and Alcohol Seeking

One of the major issues encountered in the treatment of alcohol addiction is relapse following abstinence. In alcoholic individuals, abstinence is accompanied by craving, a strong desire to engage in alcohol drinking often referred to as alcohol seeking behavior, which is in turn responsible for relapse (Martin-Fardon and Weiss 2013; Everitt and Robbins 2000; Le and Shaham 2002). Craving is typically triggered by a number of different factors, of which the most common are exposure to stress, exposure to alcohol (i.e., priming), and exposure to conditioned environmental stimuli previously associated with alcohol (i.e., conditioned cues). In this chapter, we focus on seeking behavior triggered by exposure to either priming or alcohol conditioned cues, as they are factors triggering relapse studied in relation to SigR system.

7.1 Priming-Induced Alcohol Seeking Behavior

In alcoholics, relapse and craving during abstinence are often triggered by acute reexposure to alcohol (Chutuape et al. 1994; Hodgson et al. 1979). Small amounts of alcohol can act much like *hors d'oeuvres*, thereby contributing to the "first-drink" relapse phenomenon (Ludwig et al. 1974). Literature suggests that SigRs are involved in the mechanisms underlying priming-induced alcohol seeking behavior. Indeed, Bhutada and colleagues examined the effects of SigR ligands on priming-induced reinstatement of ethanol conditioned place preference (Bhutada et al. 2012). This procedure is based on the conditioned place paradigm described previously. Briefly, specific tactile and visual stimuli of one of the two compartments of a place preference apparatus are associated with the effects of alcohol, while the stimuli of the other compartment remain neutral. Once ethanol place preference has been established, subjects are repeatedly exposed to the alcohol-paired compartment until preference is gradually extinguished. Once the alcohol preference is extinguished, it can be reinstated by exposure to alcohol or to another pharmacological agent (i.e., cross-reinstatement). In this study, the authors demonstrated that alcohol seeking behavior could be reinstated by systemic administration of 1 g/kg of ethanol or cross-reinstated by intracerebroventricular microinfusion of the selective Sig-1R PRE-084 (1–10 μg/mouse). In addition, the selective Sig-1R antagonist BD1047 (1–10 μg/mouse), microinfused intracerebroventricularly, was able to dose dependently block both ethanol-induced reinstatement and the PRE-084-induced cross-reinstatement of ethanol-induced conditioned place preference, suggesting that reinstatement of ethanol conditioned place preference involves the activation of central Sig-1Rs (Bhutada et al. 2012).

7.2 Cue-Induced Alcohol Seeking Behavior

As previously mentioned, once contextual stimuli are associated with the positive effects of alcohol through Pavlovian conditioning, they can exert a strong control over behavior. These conditioned cues become particularly relevant in occasions in which the effects of alcohol are not being experienced (i.e., during abstinence), and can lead to resumption of alcohol drinking. In preclinical psychopharmacological research, different animal models of alcohol seeking behavior have been developed to study the influence of stimuli associated with alcohol. Here we will be describing two operant responding alcohol seeking procedures, which have been used to assess the role of SigRs in the modulation of the influence of alcohol-associated cues over behavior.

A classical experimental procedure used to assess seeking behavior is the cue-induced reinstatement of seeking behavior. In this task, subjects are trained to self-administer alcohol by pressing a lever, and each lever response is contiguously paired with a brief presentation of a conditioned stimulus (e.g., an olfactory

stimulus, a light, a tone). Following the initial training, ethanol-reinforced responding is extinguished by withholding both alcohol delivery and presentation of the conditioned stimulus. Once extinction of lever responding is obtained, reinstatement of alcohol seeking behavior is induced by presenting the alcohol-associated conditioned stimulus. Using this procedure, Martin-Fardon and colleagues showed that the selective Sig-1R BD1047 (3–20 mg/kg) was able to block cue-induced reinstatement of alcohol seeking induced by presentation of an olfactory stimulus.

Another classical experimental procedure used to assess seeking behavior is the seeking-taking chain in a second-order schedule of reinforcement, where responding on a seeking lever is maintained not only by the self-administered reinforcer, but also by contingent presentation of reinforcer-paired stimuli that serve as conditioned reinforcers of instrumental behavior (Velazquez-Sanchez et al. 2015; Everitt and Robbins 2000; Giuliano et al. 2015). Typically, a second inactive lever is present and responses on this lever result in no consequences, but are recorded as an index of motor activity. This procedure has been recently established employing alcohol as the reinforcer, and it has been used to determine the role of SigR in alcohol seeking behavior (Blasio et al. 2015). The selective Sig-1R antagonist BD1063 (3–30 mg/kg) systemically administered was shown to be able to dramatically and dose dependently reduce alcohol seeking behavior. All doses of BD1063 tested significantly decreased the number of lever presses and importantly BD1063 did not affect responding on the inactive lever, ruling out an overall behavioral suppression.

Altogether, these data suggest that the ability of alcohol-associated cues to induce seeking behavior involves the activation of Sig-1R.

8 Sigma Receptors and Cognitive Impairment During Alcohol Withdrawal

Withdrawal from chronic consumption of alcohol is characterized by a plethora of physical, motivational, cognitive, and emotional symptoms (Pitel et al. 2007; Beatty et al. 1995; McKeon et al. 2008; Koob 2003). Withdrawal symptoms can be unpleasant and intense, and can develop from several hours to a few days after the cessation (or reduction) of heavy and prolonged alcohol use; while certain symptoms may be short lasting, others can persist for months and contribute to relapse (Koob 2000, 2003; American Psychiatric Association 2013).

The impairment in cognitive function is a symptom associated with chronic alcohol exposure withdrawal (Beatty et al. 1995; Pitel et al. 2007), and has been demonstrated to involve the Sig-1R system (Meunier et al. 2006; Sabeti 2011; Sabeti and Gruol 2008). In a study conducted by Meunier and colleagues, mice were shown to develop cognitive dysfunction in a novel object recognition task, during a 16-day withdrawal period which followed 4 months of chronic alcohol consumption. In this task, mice were tested for their ability to habituate to familiar objects, to correctly locate familiar object in different spatial locations, and to recognize familiar vs. novel

objects. Alcohol-withdrawn mice showed increased locomotion, anxiety, and object exploration, which impeded correct reaction to object habituation, spatial change, and novelty. Importantly the authors showed that treatment with either a nonselective Sig-1R agonist (igmesine) or a Sig-1R antagonist (BD1047) restored correct reactions to spatial change and novelty in mice (Meunier et al. 2006). In addition, these mice had upregulated Sig-1R expression in the hippocampus, which was attenuated following repeated administration of either Sig-1R ligand, suggesting that the increase in hippocampal Sig-1R levels may mediate the ethanol withdrawal-induced cognitive impairments (Meunier et al. 2006).

In addition, it was shown using slice electrophysiology that withdrawal from chronic intermittent ethanol vapors during adolescence significantly alters long-term potentiation in the hippocampus via a Sig-1R-related mechanism (Sabeti and Gruol 2008; Sabeti 2011). In a first study, authors examined how chronic ethanol exposure during adolescence affects long-term potentiation (LTP) mechanisms in the hippocampus (Sabeti and Gruol 2008). The study shows that the selective Sig-1R antagonist BD1047 blocked a slow-developing NMDAR-independent LTP in excitatory CA1 synapses in hippocampal slices at 24 h after CIE vapor exposure. In addition, in alcohol-withdrawn early-adolescent animals, authors observed a Sig-1R-dependent increased presynaptic function during NMDAR-independent LTP induction.

In a second study, the same authors found that, in slices obtained from adolescent rats exposed to chronic intermittent alcohol, CA1 neurons responded to the induction of large-amplitude LTP stimulations with a reduced excitability during ethanol withdrawal compared to slices obtained from ethanol-naïve rats. Importantly these impairments, which manifested as decreased spike efficacy and impaired activity-induced field excitatory postsynaptic potential-to-spike (E-S) potentiation, were normalized by the Sig-1R antagonist BD1047. These data suggest that acute ethanol withdrawal recruits Sig-1Rs, which in turn act to depress the efficacy of excitatory inputs in triggering action potentials during LTP.

9 Concluding Remarks

As reviewed above, there is growing evidence that the Sig-1R system may represent a novel target for the pharmacological treatment of alcohol-use disorders. Sig-1R antagonists have proven effective in reducing excessive alcohol drinking and alcohol seeking behavior in multiple animal models, suggesting that Sig-1R activation mediates the susceptibility to drink high quantities of alcohol. However, the exact mechanisms through which the Sig-1R system influences the actions of alcohol are still not entirely clear. Therefore, mechanistic studies aimed at understanding the interaction between the Sig-1R system and alcohol are warranted to improve our understanding of the neurobiological bases of alcoholism and help develop novel therapeutic options for this disorder.

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Sigma1 Pharmacology in the Context of Cancer

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Abstract

Sigma1 (also known as sigma-1 receptor, Sig1R, σ1 receptor) is a unique pharmacologically regulated integral membrane chaperone or scaffolding protein. The majority of publications on the subject have focused on the neuropharmacology of Sigma1. However, a number of publications have also suggested a role for Sigma1 in cancer. Although there is currently no clinically used anticancer drug that targets Sigma1, a growing body of evidence supports the potential of Sigma1 ligands as therapeutic agents to treat cancer. In preclinical models, compounds with affinity for Sigma1 have been reported to inhibit cancer cell proliferation and survival, cell adhesion and migration, tumor growth, to alleviate cancer-associated pain, and to have immunomodulatory properties. This review will highlight that although the literature supports a role for Sigma1 in cancer, several fundamental questions regarding drug mechanism of action and the physiological relevance of aberrant *SIGMAR1* transcript and Sigma1 protein expression in certain cancers remain unanswered or only partially answered. However, emerging lines of evidence suggest that Sigma1 is a component of the cancer cell support machinery, that it facilitates protein interaction networks, that it allosterically modulates the activity of its associated proteins, and that Sigma1 is a selectively multifunctional drug target.

Keywords

Allosteric modulation • Cancer • Cancer pain • Chaperone • Context • Drug mechanism of action • Immunomodulation • Lipid • Metabolism • Modulator • Multifunctional drug target • Protein homeostasis • Protein–protein interaction • Scaffold • Sigma1 • Sigma-1 receptor • Small molecule

1 Introduction

Sigma1 shares no significant homology with any other proteins encoded in the human genome (Hanner et al. 1996; Schmidt et al. 2016). Historically it has been considered a receptor. However, emerging evidence suggests that Sigma1 functions as a novel pharmacologically regulated integral membrane chaperone or scaffolding protein (Hayashi and Su 2007; Crottes et al. 2011, 2016; Thomas et al. 2017). Consistent with this notion, Sigma1 is involved in aspects of cellular protein homeostasis including protein synthesis, folding, trafficking, and degradation (Kim et al. 2012; Hayashi and Su 2007; Crottes et al. 2011, 2016; Schrock et al. 2013; Thomas et al. 2017).

Although most publications regarding Sigma1 describe it in the context of neuropharmacology (Cobos et al. 2008; Maurice and Su 2009), a number of publications over the years have described a potential role for Sigma1 in cancer biology. Until recently, this relationship has been largely based on two lines of

evidence: (1) reports of elevated expression levels of Sigma1 protein and *SIGMAR1* transcripts in some cancer cell lines and some tumors (reviewed in Sects. 2 and 3, below); and (2) antiproliferative and growth inhibiting effects of some small molecule inhibitors (putative antagonists) of Sigma1 on cancer cell lines (reviewed in Sect. 4, below, and Table 1). However, despite well over a hundred publications directly addressing the subject, the physiological role of Sigma1 in cancer cells remains poorly understood.

There is no compelling evidence that *SIGMAR1* is an oncogene or that Sigma1 is an oncogenic driver protein. However, several studies have demonstrated that cancer cells require functional, intact Sigma1 to grow, proliferate, and survive. Sigma1 RNAi and some small molecule inhibitors (putative antagonists) of Sigma1 have been reported to inhibit cell growth, proliferation, and cell survival. Conversely, increased Sigma1 protein levels through overexpression of recombinant Sigma1 and enhancing Sigma1 with small molecule activators (putative agonists) have been reported to promote some of these processes in cancer cells (reviewed in Sects. 4 and 6, below).

Most of our knowledge of Sigma1 comes from pharmacological studies that have implicated this protein in multiple cellular processes including control of apoptosis, cell cycle, cell growth, proliferation, endoplasmic reticulum (ER) stress, protein and lipid homeostasis, autophagy, and ion channel regulation (reviewed in Sects. 4–6, below). As it was originally identified as a receptor, small molecules with affinity for Sigma1, so-called Sigma1 ligands, have been classified as agonists and antagonists. These are evolving concepts, and in light of emerging data these definitions may not be accurate given that Sigma1 is not a bona fide receptor. We propose that the term modulator may be more appropriate for compounds with affinity for Sigma1. However, in this review we will continue to use the terms ligand/modulator, antagonist/inhibitor, and agonist/activator in order to integrate the decades of published data on the pharmacology of Sigma1 in cancer (see Sect. 4, below).

Several review articles have broadly surveyed compounds with affinity for Sigma1 and have described their effects on cancer cell lines (Abate 2012; Megalizzi et al. 2012; van Waarde et al. 2015; Brust et al. 2014). We have listed the published Sigma1 associated functional activities and binding affinities of many of these compounds in Tables 1 and 2. In this review, we will focus on a number of salient examples of how putative Sigma1 ligands have been used in cancer cell lines and what they reveal about Sigma1 biology in the context of cancer. We will review the historical classification of Sigma1 modulators as activators and inhibitors (putative agonists and antagonists), the cellular pathways and processes engaged by Sigma1 modulator compounds, the immunomodulatory effects of these compounds, and their potential as agents to treat cancer-associated comorbidities such as cancer pain as well as inhibit tumor growth (see Sects. 4–6, below). We will also review evidence from clinical trials as well as preclinical animal studies showing that the on-target effects of Sigma1 modulators do not produce adverse effects.

Table 1 Sigma ligands tested in cancer cell line studies (presented in chronological order of publication)

Reference	Compound name	Cell lines tested	Assays used	Results	MOA (proposed)
(Vilner and Bowen 1993)	Haloperidol, reduced haloperidol, fluphenazine, perphenazine, pimozide, spiperone	C6 rat glioma	Scoring of morphological changes	Loss of processes, discontinued cell division, eventual cell death	Not specified
(Vilner et al. 1995a, b)	Haloperidol, reduced haloperidol, fluphenazine, perphenazine, trifluoperazine, BD737, LR172, BD1008, SH-344, trifluperidol, thioridazine, (−)-butaclamol	C6 rat glioma, SK-N-SH, SH-SY5Y, NCB-20, NG108-15, PC12	Scoring of morphological changes, trypan blue exclusion to confirm score	Loss of processes, discontinued cell division, eventual cell death (dependent on time, dose, and pH)	Not specified
(Brent and Pang 1995)	Haloperidol, reduced haloperidol, DTG, (+)-pentazocine, (−)-pentazocine, rimcazole, (+)- and (−)-*N*-allylnormetazocine (SKF 10047)	MCF-7, LIM 1215, WIDr, melanoma (Chinnery)	MTT assay	Inhibition of cell proliferation, cell detachment, rounding of cells	Not specified
(Brent et al. 1996)	Reduced haloperidol	MCF-7, WIDr	Nuclear staining with Hoechst 33258, cellular DNA fragmentation ELISA, condensation of heterochromatin using transmission electron microscopy, FURA-2/AM calcium assay	Inhibition of cell proliferation, cell death	Induction of apoptosis, potentially through an increase in intracellular calcium
(Labit-Le Bouteiller et al. 1998)	SR31747A	Jijoye, U937, HL60, TF1, MCF-7, B9, CTLL2, M1, COS, CHO	MTT assay, gas chromatography, mass spectroscopy	Inhibition of cell proliferation, reversible by cholesterol	Inhibition of cholesterol biosynthesis via emopamil-binding protein
(John et al. 1999)	PIMBA	DU145, LNCaP, PC3	Soft agar colony formation assay	Inhibition of colony formation	Not specified
(Moody et al. 2000)	2-IBP (xenografts), IPAB (xenografts), haloperidol	NCI-H209, NCI-H345, NCI-N417 (xenografts)	MTT assay, soft agar colony formation assay, tumor xenografts	Inhibition of cell proliferation, inhibition of tumor xenograft growth	Not specified
(Crawford and Bowen 2002)	CB-64D, CB-184, haloperidol, reduced haloperidol, CB-184 in combination with doxorubicin or actinomycin D, haloperidol in combination with doxorubicin	MCF-7, T47D, MCF-7/ADR, SKBR3	CytoTox 96 kit to measure lactate dehydrogenase (LDH) release, TUNEL staining, ApoAlert annexin-V apoptosis kit	Cell death, potentiation of cytotoxicity with combination treatments	Novel p53- and caspase-independent apoptosis

(Berthois et al. 2003)	SR31747A	MCF-7 (xenografts), MDA-MB-231 (xenografts), LNCaP (xenografts), DU145 (xenografts), PC3 (xenografts), BT20	MTT assay, tumor xenografts	Inhibition of cell proliferation, inhibition of tumor xenograft growth (in combination with tamoxifen in MCF-7)	Potentially via binding to EBP or other binding site
(Barbieri et al. 2003)	Five (1α/1β-arylalkyl) quinolizidines including two thioisosteres and four spiro-[3,4-dihydro-1,2,4-benzotriazino-3,4′-(1′-substituted) piperidines]: ANS-1, ANS-2, ANS-3, ANS-4, ANS-5, FN/C-1, FN/C-2, FN/C-3, FN/C-4	MCF-7, MDA-MB-231	MTT assay	Inhibition of cell growth, cytotoxicity	Not specified
(Ferrini et al. 2003)	SR31747A	PC3, DU145, MDA-MB-231	CellTiter 96 Aqueous cell proliferation assay kit, DNA microarray, northern blot, affymetrix HC-G110 cancer gene array	Inhibition of cell proliferation	Decrease in expression of genes involved in DNA replication and progression of cell cycle, decrease in expression of 3 enzymes (dihydrofolate reductase, thymidylate synthase, thymidine kinase) involved in nucleotide synthesis
(Colabufo et al. 2004)	PB-28, NE-100, DTG, haloperidol, (+)-pentazocine	SK-N-SH, C6 rat glioma	MTT assay, CytoTox-One cytotoxicity assay (lactate dehydrogenase release), Apo-One homogenous caspase-3/7 kit	Inhibition of cell proliferation, cytotoxicity	Caspase-independent apoptosis
(Spruce et al. 2004)	Rimcazole (xenografts), IPAG (xenografts), reduced haloperidol, haloperidol (xenografts), BD1047, BD1063, *cis*-U50488 (xenografts)	MDA-MB-468 (xenografts), MDA-MB-435 (xenografts), MCF-7 (xenografts), H1299 (xenografts), PC3M (xenografts)	MTS CellTitre proliferation assay, colony formation assay, Apo-one homogeneous caspase3/7 assay, flow cytometry, FURA-2/AM calcium assay, tumor xenografts	Inhibition of cell survival, inhibition of xenograft growth	Caspase-dependent apoptosis, rise in cytosolic calcium, activation of phospholipase C, inhibition of PI3K pathway
(Renaudo et al. 2004)	(+)-Pentazocine, igmesine, DTG	NCI-H209, NCI-H146, Jurkat	Whole-cell patch-clamp, trypan blue exclusion, DEVD-pNA cleavage assay to analyze caspase activity, DNA fragmentation	Inhibition of cell growth (Jurkat, igmesine)	Inhibition of K^+ channel, accumulation of $p27^{kip1}$, not apoptosis

(continued)

Table 1 (continued)

Reference	Compound name	Cell lines tested	Assays used	Results	MOA (proposed)
(Wang et al. 2004)	Haloperidol, reduced haloperidol, progesterone, combination of reduced haloperidol + doxorubicin, vinorelbine, paclitaxel, and docetaxel	MDA-MB-231, MDA-MB-361, MDA-MB-435, MCF-7, BT20	CellTiter 96 Aqueous One cell proliferation assay	Growth inhibition, additive effect of growth inhibition with reduced haloperidol and chemotherapy combinations	Not specified
(Ostenfeld et al. 2005)	Siramesine	WEHI-S, WEHI-R4 (xenografts), MCF-7, MCF-7S1 (xenografts), MDA-MB-468, HeLa, ME-180	MTT assay, lactate dehydrogenase release, flow cytometry, caspase activity measurement, tumor xenografts	Cell death, cell shrinkage and detachment, inhibition of xenograft growth	Increase in reactive oxygen species (ROS), permeabilization of the lysosomal membrane
(Nordenberg et al. 2005)	Haloperidol, reduced-haloperidol, ifenprodil tartrate, opipramol, carbetapentane citrate, haloperidol in combination with imatinib mesylate (STI 571)	B16, SK-MEL-28	SRB colorimetric cytotoxicity assay, DNA fragmentation, flow cytometry, ELISA cell death assay, immunoblot, spectrofluorometric ATP measurement	Inhibition of cell growth (synergy with haloperidol and imatinib mesylate combination), G1 cell cycle arrest, decrease in cell viability	Apoptosis, decrease in ATP levels, decrease in cyclin D and CDK2 protein levels in cytoplasm and nucleus
(Azzariti et al. 2006)	PB-28, PB-28 in combination with doxorubicin	MCF-7, MCF-7 ADR	MTT assay, annexin-V staining, propidium iodide (PI) staining, flow cytometry, immunoblot, Apo-one homogenous caspase-3/7 kit	Inhibition of cell growth, increase in accumulation of intracellular doxorubicin, increase in cytotoxicity when in combination with doxorubicin when compared to doxorubicin alone	Increase in percent of cells in the G_0-G_1 phase, induction of caspase-independent apoptosis, decrease in P-gp expression
(Aydar et al. 2006)	(+)SKF10047, ibogaine	MCF-7, MDA-MB-231	Crystal violet staining assay, single-cell adhesion measuring apparatus	SKF10047 – inhibition of proliferation in MDA-MB-231 cells, reduction in adhesion in both cell lines Ibogaine – inhibition of cell proliferation in both cell lines, reduction in adhesion in both cell lines	Not specified
(Wei et al. 2006)	Haloperidol	PC12, N2a	MTT assay, lactate dehydrogenase release, flow cytometry, subcellular fractionation, immunoblot	Decrease in cell viability	Increase in Bcl-XS expression and translocation to mitochondria, apoptosis
(Geiger et al. 2007)	Stereoisomeric alcohols and methyl ethers from (R)- and (S)-glutamate	5637, RT-4, A-427, LCLC-103H, MCF-7	Microtiter assay with crystal violet staining	Inhibition of cell growth (methyl ethers > alcohols) and cell death (methyl ethers)	Not specified

(Kashiwagi et al. 2007)	SV119, WC-26 (tumor allografts), haloperidol	Panc-1, CFPAC-1, ASPC-1, Panc-02 (tumor allografts)	TUNEL staining, flow cytometry, tumor allografts	Cell death, decrease in tumor allograft growth, improved survival	Caspase-3/7-dependent apoptosis
(Megalizzi et al. 2007)	4-IBP	U373-MG (xenografts), C32, A549 (xenografts), PC3	Immunoblot, TUNEL staining, flow cytometry, colorimetric MTT assay, computer-assisted phase-contrast microscopy, fluorescence microscopy, scratch wound assay, tumor xenografts	Inhibition of proliferation, decrease in migration, increased sensitivity to proapoptotic (lomustin) and proautophagic (temozolomide) drugs, increased survival in vivo (U373-MG), increased therapeutic benefit of temozolomide (U373-MG) and IRI (A549) in vivo	Not apoptosis or autophagy, alteration to actin cytoskeleton organization, decrease in glucosylceramide synthase and Rho guanine nucleotide dissociation inhibitor (important for drug resistance)
(Achison et al. 2007)	Rimcazole	HCT-116 ($p53^{+/+}$ or $^{-/-}$), MDA-MB-231	Immunoblot, CellTiter 96 Aqueous one solution cell proliferation assay (MTS), PI staining, flow cytometry	Cell death	Increase in HIF-1α levels under normoxic conditions only in cancer cells (partly dependent on p53), apoptosis (more potent in $p53^{+/+}$ cells)
(Renaudo et al. 2007)	Igmesine, DTG, (+)-pentazocine, NPPB	NCI-H209, JA3, HEK-SIG (Sigma1 transfected HEK cells)	Trypan blue exclusion, immunoblot, electronic sizing for volume measurements with CASY 1 (SCARFE SYSTEM), whole cell patch clamp	Inhibition of cell proliferation, inhibition of cell cycle, delayed/eliminated regulatory volume decrease	Inhibition of volume-regulated chloride channels (VRCC), accumulation of p27, affected rate of activation of VRCC and cell volume regulation, which could protect cells from apoptosis
(Rybczynska et al. 2008)	Rimcazole, haloperidol	C6 rat glioma	Competition of ligand binding with ^{11}C-SA4503, measuring uptake of PET tracers to examine metabolic activity, trypan blue exclusion, morphology observations	Decrease in cell viability, increase in PET tracer uptake (^{18}F-FDG) or decrease in PET tracer uptake (^{18}F-FLT and ^{11}C-choline)	Very high occupancy of Sigma2 receptors
(Ostenfeld et al. 2008)	Siramesine	MCF-7 (xenografts), U2OS, WEHI-S	Immunoblot, immunocytochemistry, subcellular fractionation, acridine orange staining, lysotracker staining, measurement of cathepsin activity by zFR-AFC probe, flow cytometry, MTT assay, lactate dehydrogenase release, tumor xenografts	Cathepsin-dependent cell death, no increase in protein degradation, sensitization to cell death with combination of siramesine and autophagy inhibitor (3-MA)	Localization of siramesine in lysosomes, increase in lysosomal pH, inhibition of mTORC, acts as a lysosomotropic detergent that leads to destabilization of lysosomes, buildup of protective autophagosomes (in vivo and in vitro)

(continued)

Table 1 (continued)

Reference	Compound name	Cell lines tested	Assays used	Results	MOA (proposed)
(Megalizzi et al. 2009)	4-IBP, 4-IPAB, haloperidol, BD1008, eliprodil, donepezil, dextromethorphan, IPAG	Hs683 (xenografts), U373, T98G, U87, SW1783, A172, SW1088, U138, H4, U118	Computer-assisted phase-contrast videomicroscopy, scratch wound assay, global growth ratio calculations, tumor xenografts	Decrease in cell recolonization (4-IBP, donepezil, IPAG, dextromethorphan, BD1008, and haloperidol, U373 and T98G), cell death, decrease in cell division and increased survival in tumor xenografts (particularly with combination of donepezil and temozolomide)	Increase in cell mitosis duration, cell mitotic arrests
(Berardi et al. 2009)	Analogs of PB-28, such as piperidines **24** and **15**	SK-N-SH	MTT assay	Inhibition of cell proliferation	Not specified
(Kashiwagi et al. 2009)	SV119, SV119 in combination with gemcitabine and paclitaxel (allografts)	Panc-02 (allografts), CFPAC-1, Panc-1, ASPC-1	TUNEL staining, caspase-3 detection, flow cytometry, tumor allografts	Cell death, decreased tumor growth and increased survival in allografts (combination of SV119 and gemcitabine or paclitaxel)	Apoptosis (particularly when Sigma2 ligands in combination with chemotherapy) in vitro and in vivo
(Holl et al. 2009a, b, c)	6,8-Diazabicyclo[3.2.2]nonane derivatives, such as benzylidene derivatives **17** and benzyl ethers **11**	A-427	Microtiter crystal violet staining assay	Inhibition of cell growth	Not specified
(Holl et al. 2009)	Allyl and benzyl substituted 6,8-diazabicyclo[3.2.2]nonan-2-one derivatives **5**, ent-**5** and ent-**14**	5637	Microtiter crystal violet staining assay	Inhibition of cell growth	Not specified
(Holl et al. 2009)	6-Allyl-6,8-diazabicyclo[3.2.2] nonane derivatives, such as methyl ethers ent-**16b**, **21a,** ent-**21a**, and **21b,** and unsubstituted compounds **23a** and **23b,** and bicyclic acetal **11**	A-427	Microtiter crystal violet staining assay	Inhibition of cell growth	Not specified
(Piergentili et al. 2010)	Novel antagonists related to spipethiane, such as **4-10**	MCF-7, MCF-7 ADR	SRB assay, annexin-V staining, PI staining, flow cytometry	Inhibition of cell growth, induction of cell death of MCF-7 ADR (high expressers of Sigma1)	Inhibition of cell cycle, induction of apoptosis

(Hornick et al. 2010)	SW43, SV119, siramesine, sigma2 ligands in combination with gemcitabine	Panc-02 (allografts), MIA PaCa-2, Panc-1, BXPC3, CFPAC, ASPC-1	CellTiter-Glo assay, caspase-3 staining, annexin-V and PI staining, flow cytometry, image-iT live green reactive oxygen species detection kit, tumor allografts	Decrease in cell viability and decrease in tumor allograft growth (particularly when ligands in combination with gemcitabine)	Activation of caspase-3, increase in ROS, induction of apoptosis
(Sunnam et al. 2010)	Conformationally restricted ligands derived from a 7,9-diazabicyclo[4.2.2]decane scaffold, such as methyl ether **25b** and unsubstituted derivatives **26** and **27**	A-427, MCF-7, 5637	Microtiter crystal violet staining assay	Inhibition of cell growth (**25b**, **26**, and **27**)	Not specified
(Hajipour et al. 2010)	*N*,*N*-dialkyl (**1–3**,) or *N*-alkyl-*N*-aralkyl compounds (compounds **4–18**)	NCI-H460, SK-OV-3, DU145, MCF-7, SF-268, A549, MDA-MB-231, HT-29, HCT-15, H1299	Multiplex cytotoxicity assays by Keck-UWCCC small molecule screening facility	Inhibition of cell proliferation (**9**, **3**, **15, 19**, **20**), cytotoxicity	Not specified
(Ahmed et al. 2010)	AG-205	A549, MDA-MB-231, MDA-MB-468	MTT assay, immunoblot, flow cytometry	Inhibition of cell growth, decrease in cell viability	Inhibition of cell cycle, posttranscriptional increase in Sigma2, decrease in ERK phosphorylation
(Marrazzo et al. 2011a, b)	(−)-Methyl (1S,2R)-2-{[(3-endo)-3-(4-Chlorophenyl)-3-hydroxy-8-azabicyclo[3.2.1] oct-8-yl]methyl}-1-phenylcyclopro-panecarboxylate (9)	LNCaP, PC3	MTT assay	Inhibition of cell proliferation	Not specified
(Marrazzo et al. 2011a, b)	Phenylbutyrate ester of haloperidol metabolite II (±)-MRJF4	LNCaP, PC3	MTT assay	Decrease in cell viability	Not specified
(Yarim et al. 2011)	Indole scaffold based compounds **1a-c, 3a-b, 4a-b**	MCF-7, HUH7, HCT-116	TCA fixation and SRB staining	Cell death	Not specified
(Chu et al. 2011)	*N*-3-(4-nitrophenyl)propyl derivatives of heptylamine (2a and 2b), dodecylamine (3a and 3b)	MDA-MB-231, MCF-7, NCI-H460, A549, H1299, HCT-15, HT-29, SK-OV-3, DU145, SF-268	Calcein-AM, CellTiter-Glo assay, EthD-1	Cell death	Not specified
(Pal et al. 2011)	Haloperidol, cationic lipid-conjugated haloperidol HP-C4, HP-C8 (allografts), HP-C12, HP-C16	MCF-7, MDA-MB-231, ZR-75-1, B16F10 (allografts)	MTT assay, annexin-V and PI staining, TUNEL staining, flow cytometry, immunoblot	Inhibition of cell proliferation (particularly HP-C8, and more in cancer cells than normal cells), decrease in cell viability, inhibition of allograft tumor growth	Decrease in pAKT signaling, induction of caspase-3-mediated apoptosis

(continued)

Table 1 (continued)

Reference	Compound name	Cell lines tested	Assays used	Results	MOA (proposed)
(Abate et al. 2011)	Novel cyclohexylpiperazine derivatives *cis*-**7**, *trans*-**7**, *cis*-**8**, *trans*-**8**, *cis*-**9**, *trans*-**9**, *cis*-**10**, *trans*-**10**, *cis*-**11**, *trans*-**11**, *cis*-**12**, *trans*-**12**, *cis*-**13**, *trans*-**13**, *cis*-**14**, *trans*-**14**, **1**, **15**, *cis*-**11** in combination with doxorubicin	SK-N-SH, PC3, MDCK-MDR1	MTT assay, calcein-AM assay	Inhibition of cell proliferation (especially with *cis*-**11** in combination with doxorubicin), increased cell death, p-glycoprotein inhibition	Not specified
(Spitzer et al. 2012)	SV119, conjugates S2-CTMP-4, S2-rapamycin, and S2-Bim (allografts), S2-Bim in combination with gemcitabine and radiation	Panc-02 (allografts), Panc-1, ASPC-1, and CFPAC (xenografts)	TUNEL staining, caspase-3 staining, flow cytometry, tumor allografts and xenografts	Cell death (augmented by S2 conjugates), inhibition of allograft growth (S2-Bim > SV119), augmented cell death with combination of S2-Bim with gemcitabine and radiation in vitro	Disruption of intracellular signaling pathways (AKT for S2-CTMP-4, p70S6K for S2-rapamycin), apoptosis
(Kim et al. 2012)	IPAG, haloperidol	T47D, MCF-7, MDA-MB-468, LNCaP, PC3	Flow cytometry, trypan blue exclusion, BCA assay, m^7GTP-sepharose bead mimicking 5′ mRNA cap pull-down	Reversible decrease in cell mass, cell death with continuous treatment over time	Reversible decrease in cap-dependent translation initiation
(Hornick et al. 2012)	SW43 (xenografts), PB282 (xenografts), SV119 (xenografts), PB-28 (xenografts), derivatives of SW43 and PB282	BXPC3 (xenografts), Panc-02, ASPC-1	Flow cytometry, caspase-3 assay, microscopy, cellular protease assay, CellTiter-Glo assay, image-iT live Green reactive oxygen species detection kit, tumor xenografts	Cell death, inhibition of xenograft growth	Caspase-independent (SW43) or caspase-dependent (PB282) death after lysosomal membrane permeabilization, protease translocation, oxidative stress (SW43)
(Zeng et al. 2012)	WC-26, SV119, RHM-138, siramesine	EMT-6, MDA-MB-435	CellTiter96 Aqueous one MTS assay, lactate dehydrogenase release, TUNEL staining, flow cytometry, caspase-3 assay, immunoblot, transmission electron microscopy	Cell death	Induction of autophagy, mTOR inhibition, alteration of cell cycle progression, caspase activation, apoptosis
(Riganas et al. 2012a, b, c)	C1-substituted adamantine piperazines **2a** (xenografts), **2b, 2c, 2d, 2e**, **4**	NCI-H460, DMS 114, NCI-H69, H69AR, HL-60, MIA PaCa-2 (xenografts), BXPC3 (xenografts), SKHep1, LOX-IMVI, HCT-116, HCT-15, DU145, PC3, MCF-7, IGROV-1, OVCAR-5, SF268, SF295, U251	TCA fixation and SRB staining, annexin-V binding, caspase-3 assay, flow cytometry, PI staining, 7-AAD incorporation, tumor xenografts, formalin test	Inhibition of cell proliferation, cytotoxicity, inhibition of xenograft growth, analgesia (**2a**)	Caspase-3 activation, inhibition of cell cycle, apoptosis

(Riganas et al. 2012a, b, c)	Novel adamantane phenylalkylamines **2a**−**d**, **3a**−**c**, and **4a**−**e,** particularly **4a** (xenografts), **4a** in combination with 5-fluorouracil and gemcitabine (xenografts)	BXPC3 (xenografts), PC3 (xenografts), DU145 (xenografts), OVCAR-5 (xenografts), IGROV-1, HL-60 (xenografts), HCT-116, HCT-15, MCF-7, U251, SKHep1, MIA PaCa-2	TCA fixation and SRB staining, PI staining, annexin-V staining, flow cytometry, caspase-3 assay, tumor xenografts, observing auxiliary region and abdominal region for metastases and subsequent isolation and subculture to determine cell type origin, formalin test	Inhibition of cell proliferation, cytotoxicity, decreased tumor size in xenografts, synergistic anti-tumor effects with reference compounds in xenografts, decreased metastasis, analgesia (**4a**)	Caspase-3 activation, inhibition of cell cycle, apoptosis
(Riganas et al. 2012a, b, c)	4-(1-adamantyl)-4,4-diarylbutylamines **1**, 5-(1-adamantyl)-5,5-diarylpentylamines **2** and 6-(1-adamantyl)-6,6-diarylhexylamines **3**, **1a** (xenografts), **1a** in combination with paclitaxel (formalin test)	IGROV-1 (xenografts), HCT-116, HCT-15, Caki, DU145, PC3, MDA-MB-231, MCF-7, OVCAR-5, ADR-res NCI, SF268, U251, NCI-H460, DMS 114, HL-60 (TB), BXPC3, SKHep1, LOX-IMVI, SK-MEL-28, CCS WD6	TCA fixation and SRB staining, tumor xenografts, formalin test	Inhibition of cell proliferation, inhibition of xenograft growth, analgesia (**1a**)	Not specified
(Schrock et al. 2013)	IPAG	MDA-MB-468, T47D, MCF-7, PC3, Panc-1, HepG2	Trypan blue exclusion, PI staining, immunoblot, microscopy	Cell death	Unfolded protein response, autophagy, apoptosis
(Rybczynska et al. 2013)	Rimcazole	A375M	Tumor xenografts	Inhibition of xenograft growth, decrease in Ki67 staining	Not specified
(Niso et al. 2013)	Siramesine, PB-28, **4**, F281, **6**, **13**, **14**, **15**, **17**, **19**, **20**, **21**, **22**, **23**, **24**, **25, 15** in combination with doxorubicin, **25** in combination with doxorubicin	MCF-7, MCF-7 ADR, MDCK-MDR1	MTT assay, calcein-AM assay, bioluminescence ATP assay	Inhibition of cell proliferation, p-glycoprotein interaction, increased sensitization of MCF-7 ADR cells to doxorubicin (**15** and **25**), collateral sensitivity (siramesine and **25**)	Generation of more ROS and higher ATP consumption in MCF-7 ADR cells than parental cells
(Korpis et al. 2014)	Enantiomeric piperazines (S)-**4** and (R)-**4**	RPMI 8226	MTT assay, annexin-V and PI staining, caspase staining, flow cytometry	Inhibition of cell proliferation, cell death	Induction of incomplete autophagy, lipid peroxidation, altered mitochondrial membrane potential, caspase-independent apoptosis
(Korpis et al. 2014)	PB-28, haloperidol, novel hydroxyethyl piperazine-based sigma ligands such as (R)-**2b**	RPMI 8226, HL60, LCLC-103H, DAN-G, MCF-7, RT-4, A-427, 5637	MTT assay, crystal violet staining assay, annexin-V and PI staining	Inhibition of cell proliferation, cell death	Apoptosis ((R)-**2b** and combination of PB-28 with (R)-**2b**)
(Weber et al. 2014)	Hydroxyethyl substituted piperazines (**7c**)	RPMI 8226, 5637, A-427, MCF-7	MTT assay, annexin-V and PI staining	Inhibition of cell growth, cell death	Apoptosis

(continued)

Table 1 (continued)

Reference	Compound name	Cell lines tested	Assays used	Results	MOA (proposed)
(Garg et al. 2014)	SW IV-134 (SMAC mimetic conjugate)	SKOV3 (xenografts), OVCAR-3, HEY A8, HEY A8 MDR	Annexin-V staining, flow cytometry, immunoblot, CellTiter-Glo assay, caspase-Glo assay systems, ELISA, qRT-PCR, tumor xenografts	Cell death, decrease in tumor burden (xenografts), increase in survival (xenografts)	cIAP-1 and cIAP-2 degradation, activation of NF-κB, TNFα-dependent cell death, caspase-dependent apoptosis
(Zeng et al. 2014)	Azabicyclononane analogs SV119, SV166, WC-26, 2b, YUN245; tropane analog RHM-138; siramesine analog siramesine	EMT-6, MDA-MB-435	MTS assay, caspase-3 activation assay	Cell death	Caspase-3 activation
(Fytas et al. 2015)	Novel 1-(2-aryl-2-adamantyl) piperazine derivatives **6-15** (particularly **6** and **13**)	HeLa, MDA-MB-231, MIA PaCa-2, NCI HI975	MTT assay	Decrease in percent cell survival	Not specified
(Nicholson et al. 2015)	CM572	SK-N-SH, MCF-7, Panc-1	MTT assay, FURA-2/AM calcium assay, immunoblot	Irreversible cell death	Increase in cytosolic calcium, cleavage of Bid
(Happy et al. 2015)	Rimcazole, in combination with Ad.p53	MCF-7, T47D, MDA-MB-231, MDA-MB-157	MTT assay, annexin-V and PI staining, flow cytometry, DCFH-DA staining, immunoblot	Cell growth inhibition, cell death, synergistic anti-tumor effect with Ad.p53	Combination: Increase in ER stress, activation of the p38 MAPK pathway, increase in ROS, increase in Bax and activated caspase-3, induction of apoptosis
(Sozio et al. 2015)	(*R*)-(+)-MRJF4 and (*S*)-(−)-MRJF4	C6 rat glioma	Annexin-V and PI staining, MTT assay, transwell chamber migration assay, flow cytometry	Inhibition of cell proliferation, decrease in migration, cell death	Late apoptosis/necrosis, increase in percent of cells in S phase
(Das et al. 2016)	(+)-SKF10047	DU145, PC3, LNCaP	MTT assay, annexin-V binding assay	Reduction in tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) killing	Sigma1 plays a role in activation of caspase-3 and caspase-8 after TRAIL
(Nicholson et al. 2016)	CM764	SK-N-SH, MG-63	MTT assay, CyQUANT cell proliferation assay, FURA-2/AM calcium assay, NAD^+/NADH quantification colorimetric kit, ATP colorimetric/fluorometric assay kit, DCFDA stain	Increase in MTT reduction without an increase in DNA replication or proliferation	Increase in cytosolic calcium, increase in NAD^+/NADH, increase in levels of ATP, reduction in ROS, increase in VEGF and HIF1α, potential induction of glycolysis
(Zampieri et al. 2016)	Novel 1-(4-(aryl(methyl)amino) butyl)-heterocyclic ligands such as **1a** and **1d**	SH-SY5Y	MTT assay	Cytotoxicity	Not specified
(Thomas et al. 2017)	IPAG, CT-189 (xenograft)	LAPC4, LNCaP, 22Rv1 (xenograft), VCaP, C4-2	Soft agar colony formation assay, crystal violet staining assay, trypan blue exclusion, immunoblot, tumor xenograft	Suppression of cell growth and survival, inhibition of xenograft growth	Proteasomal degradation of androgen receptor and androgen receptor splice variants

Table 2 Sigma ligand binding affinities

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
(+)-3-PPP	Sigma1			
	• $K_i = 86$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 109$ nM	Rat kidney	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 102$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 75$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Cobos et al. 2005)
	Sigma2			
	• $K_i = 138$ nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 108$ nM	Rat kidney	[^{3}H]DTG	(Hellewell et al. 1994)
4-IBP	Sigma1			
	• $K_i = 1.7$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(John et al. 1995a, b)
	• $K_i = 2.6$ nM	Sf9 cells	[^{3}H](+)-pentazocine	(Schmidt et al. 2016)
	Sigma2			
	• $K_i = 25$ nM	Rat liver	[^{3}H]DTG	(John et al. 1995a, b)
(+)-Pentazocine	Sigma1			
	• $K_i = 3.1$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Vilner and Bowen 1993)
	• $K_i = 5.3$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 2.2$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Geiger et al. 2007)
	• $K_i = 5.5$ nM	Rat cerebellum	[^{3}H](+)-pentazocine	(Ishiwata et al. 2006)
	• $K_i = 2.5$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Choi et al. 2001)
	• $K_i = 3.3$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Berardi et al. 2009)
	• $K_i = 4.2$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Holl et al. 2009a, b, c)
	• $K_i = 5.6$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Sunnam et al. 2010)
	• $K_i = 2.8$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Abate et al. 2011)
	• $K_i = 3.4$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Niso et al. 2013)
	• $K_i = 5.4$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Weber et al. 2014)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	• $K_i = 36.0$ nM	RPMI 8226 cells	[^{3}H](+)-pentazocine	(Weber et al. 2014)
	• $K_i = 25.8$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 15.4$ nM	Rat kidney	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 16.7$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 4.4$ nM	BE(2)-C cells	[^{3}H](+)-pentazocine	(Ryan-Moro et al. 1996)
	Sigma1			
	• $K_d = 5.8$ nM	DU145 cells	Saturation binding	(John et al. 1999)
	• $K_d = 23.1$ nM	SK-N-SH cells	Saturation binding	(Colabufo et al. 2004)
	• $K_d = 4.7$ nM	C6 rat glioma cells	Saturation binding	(Colabufo et al. 2004)
	• $K_d = 7.5$ nM	Rat liver	Saturation binding	(Hellewell et al. 1994)
	• $K_d = 23.3$ nM	Rat kidney	Saturation binding	(Hellewell et al. 1994)
	• $K_d = 7.1$ nM	MCF-7 cells	Saturation binding	(Azzariti et al. 2006)
	• $K_d = 3.9$ nM	MCF-7/ ADR cells	Saturation binding	(Azzariti et al. 2006)
	Sigma2			
	• $K_i = 2{,}470$ nM	Rat liver	[^{3}H]DTG	(Ishiwata et al. 2006)
	• $K_i = 1{,}923$ nM	Rat liver	[^{3}H]DTG	(Choi et al. 2001)
	• $K_i = 1{,}542$ nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 2{,}018$ nM	Rat liver	[^{3}H](+)-3-PPP	(Hellewell et al. 1994)
	• $K_i = 3{,}475$ nM	Rat kidney	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 6{,}611$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
(−)-Pentazocine	Sigma1			
	• $K_i = 807$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 39$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	• $K_i = 41$ nM	Rat kidney	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 40$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	Sigma2			
	• $K_i = 2{,}324$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 37$ nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 42$ nM	Rat kidney	[^{3}H]DTG	(Hellewell et al. 1994)
(+)-SKF10047	Sigma1			
	• $K_i = 597$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 54$ nM	BE(2)-C cells	[^{3}H](+)-pentazocine	(Ryan-Moro et al. 1996)
	• $K_i = 101$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 153$ nM	Rat kidney	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 420$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	Sigma2			
	• $K_i = 39{,}740$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 11{,}170$ nM	Rat liver	[^{3}H](+)-3-PPP	(Hellewell et al. 1994)
	• $K_i = 154{,}335$ nM	Rat kidney	[^{3}H]DTG	(Hellewell et al. 1994)
(−)-SKF10047	Sigma1			
	• $K_i = 50{,}399$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 1{,}339$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 2{,}366$ nM	Rat kidney	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 1{,}917$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	Sigma2			
	• $K_i = 41{,}461$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 2{,}659$ nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 2{,}929$ nM	Rat kidney	[^{3}H]DTG	(Hellewell et al. 1994)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
BD737	Sigma1			
	• $K_i = 9$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 8$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 2$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	Sigma2			
	• $K_i = 68$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 96$ nM	Rat liver	[^{3}H](+)-3-PPP	(Hellewell et al. 1994)
BD1008	Sigma1			
	• $K_i = 1$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 2$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 1$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 2$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Berardi et al. 2001)
	Sigma2			
	• $K_i = 32$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 8$ nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 83$ nM	Rat liver	[^{3}H]DTG	(Berardi et al. 2001)
BD1047	Sigma1			
	• $K_i = 0.6$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 0.9$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Matsumoto et al. 1995)
	• $K_i = 1.9$ nM	Mouse brain	[^{3}H](+)-pentazocine	(Entrena et al. 2009)
	• $K_i = 5.3$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Cobos et al. 2005)
	Sigma2			
	• $K_i = 47$ nM	Rat liver	[^{3}H]DTG	(Matsumoto et al. 1995)
BD1063	Sigma1			
	• $K_i = 7$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 4$ nM	Mouse brain	[^{3}H](+)-pentazocine	(Entrena et al. 2009)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	• $K_i = 16$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Cobos et al. 2005)
	• $K_i = 4$ nM	Mouse brain	[^{3}H](+)-pentazocine	(Nieto et al. 2012)
	• $K_i = 9$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Matsumoto et al. 1995)
	Sigma2			
	• $K_i = 449$ nM	Rat liver	[^{3}H]DTG	(Matsumoto et al. 1995)
CB-64D	Sigma1			
	• $K_i = 5{,}304$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 3{,}063$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Bowen et al. 1995)
	Sigma2			
	• $K_i = 61$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 17$ nM	Rat liver	[^{3}H]DTG	(Bowen et al. 1995)
CB-64L	Sigma1			
	• $K_i = 102$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 11$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Bowen et al. 1995)
	Sigma2			
	• $K_i = 759$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 154$ nM	Rat liver	[^{3}H]DTG	(Bowen et al. 1995)
CB-184	Sigma1			
	• $K_i = 7{,}436$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Bowen et al. 1995)
	Sigma2			
	• $K_i = 13$ nM	Rat liver	[^{3}H]DTG	(Bowen et al. 1995)
CM764	Sigma1			
	• $K_i = 87$ nM	Rat liver	[^{3}H](+)-pentazocine	(Nicholson et al. 2016)
	Sigma2			
	• $K_i = 4$ nM	Rat liver	[^{3}H]DTG	(Nicholson et al. 2016)
DTG	Sigma1			
	• $K_i = 203$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 60$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	• $K_i = 45$ nM	Rat kidney	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 51$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 69$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Marrazzo et al. 2011a)
	• $K_i = 71$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Zampieri et al. 2016)
	Sigma2			
	• $K_i = 58$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 13$ nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 22$ nM	Rat kidney	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 23$ nM	Guinea pig brain	[^{3}H]DTG	(Marrazzo et al. 2011a)
	• $K_i = 54$ nM	Rat liver	[^{3}H]DTG	(Zampieri et al. 2016)
Haloperidol	Sigma1			
	• $K_i = 4$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Vilner and Bowen 1993)
	• $K_i = 2$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 2$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Geiger et al. 2007)
	• $K_i = 3$ nM	Rat cerebellum	[^{3}H](+)-pentazocine	(Ishiwata et al. 2006)
	• $K_i = 4$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Holl et al. 2009a, b, c)
	• $K_i = 6$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Sunnam et al. 2010)
	• $K_i = 2$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Marrazzo et al. 2011a)
	• $K_i = 2$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Marrazzo et al. 2011b)
	• $K_i = 7$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Weber et al. 2014)
	• $K_i = 40$ nM	RPMI 8226 cells	[^{3}H](+)-pentazocine	(Weber et al. 2014)
	• $K_i = 3$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Sozio et al. 2015)
	• $K_i = 7$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Zampieri et al. 2016)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	• $K_i = 1$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Choi et al. 2001)
	• $K_i = 2$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 8$ nM	Rat kidney	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 6$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	Sigma2			
	• $K_i = 78$ nM	Rat liver	[^{3}H]DTG	(Geiger et al. 2007)
	• $K_i = 167$ nM	Rat liver	[^{3}H]DTG	(Ishiwata et al. 2006)
	• $K_i = 78$ nM	Rat liver	[^{3}H]DTG	(Holl et al. 2009a, b, c)
	• $K_i = 78$ nM	Rat liver	[^{3}H]DTG	(Sunnam et al. 2010)
	• $K_i = 16$ nM	Guinea pig brain	[^{3}H]DTG	(Marrazzo et al. 2011a)
	• $K_i = 16$ nM	Guinea pig brain	[^{3}H]DTG	(Marrazzo et al. 2011b)
	• $K_i = 78$ nM	Rat liver	[^{3}H]DTG	(Weber et al. 2014)
	• $K_i = 200$ nM	RT-4 cells	[^{3}H]DTG	(Weber et al. 2014)
	• $K_i = 18$ nM	Guinea pig brain	[^{3}H]DTG	(Sozio et al. 2015)
	• $K_i = 78$ nM	Rat liver	[^{3}H]DTG	(Zampieri et al. 2016)
	• $K_i = 38$ nM	Rat liver	[^{3}H]DTG	(Choi et al. 2001)
	• $K_i = 12$ nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 18$ nM	Rat liver	[^{3}H](+)-3-PPP	(Hellewell et al. 1994)
	• $K_i = 42$ nM	Rat kidney	[^{3}H]DTG	(Hellewell et al. 1994)
	• $K_i = 221$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
Haloperidol (reduced)	Sigma1			
	• $K_i = 5$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Vilner and Bowen 1993)
	• $K_i = 3$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 47$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	Sigma2			
	• $K_i = 123$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
Haloperidol–metabolite II	Sigma1			
	• $K_i = 5$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Marrazzo et al. 2011a)
	• $K_i = 2$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Marrazzo et al. 2011b)
	Sigma2			
	• $K_i = 1$ nM	Guinea pig brain	[^{3}H]DTG	(Marrazzo et al. 2011a)
	• $K_i = 1$ nM	Guinea pig brain	[^{3}H]DTG	(Marrazzo et al. 2011b)
	• $K_i = 2$ nM	Guinea pig brain	[^{3}H]DTG	(Sozio et al. 2015)
Igmesine	Sigma1			
	• $IC_{50} = 39$ nM	Rat brain	[^{3}H](+)-SKF10047	(Roman et al. 1990)
IPAG	Sigma1			
	• $K_d = 3$ nM	MDA-MB-468 cells	[^{125}I]IPAG saturation	(Schrock et al. 2013)
	• $K_d = 3$ nM	Guinea pig brain	[^{125}I]IPAG saturation	(Wilson et al. 1991)
LR172	Sigma1			
	• $K_i = 6$ nM	SK-N-SH cells	[^{3}H](+)-pentazocine	(Vilner and Bowen 2000)
	• $K_i = 1$ nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• $K_i = 0.5$ nM	C6 glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 0.4$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(McCracken et al. 1999)
	Sigma2			
	• $K_i = 14$ nM	SK-N-SH cells	[^{3}H]DTG	(Vilner and Bowen 2000)
	• $K_i = 2$ nM	Rat liver	[^{3}H]DTG	(McCracken et al. 1999)
NE-100	Sigma1			
	• $K_i = 15$ nM	Mouse brain	[^{3}H](+)-pentazocine	(Marrazzo et al. 2011a)
	• $K_i = 13$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Cobos et al. 2005)
	• $K_i = 1$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Berardi et al. 2001)
	• $K_d = 1$ nM	Guinea pig brain	[^{3}H]NE-100 saturation	(Tanaka et al. 1995)
	• $IC_{50} = 85$ nM	Guinea pig brain	[^{3}H]DTG	(Chaki et al. 1994)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	• $IC_{50} = 1$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Chaki et al. 1994)
	Sigma2			
	• $K_i = 212$ nM	Rat liver	[^{3}H]DTG	(Berardi et al. 2001)
PB-28	Sigma1			
	• $K_i = 13$ nM	MCF-7 cells	[^{3}H](+)-pentazocine	(Azzariti et al. 2006)
	• $K_i = 10$ nM	MCF-7/ ADR cells	[^{3}H](+)-pentazocine	(Azzariti et al. 2006)
	• $K_i = 0.4$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Niso et al. 2013)
	• $K_i = 14$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Berardi et al. 2004)
	Sigma2			
	• $K_i = 0.28$ nM	MCF-7 cells	[^{3}H]DTG	(Azzariti et al. 2006)
	• $K_i = 0.17$ nM	MCF-7/ ADR cells	[^{3}H]DTG	(Azzariti et al. 2006)
	• $K_i = 0.68$ nM	Rat liver	[^{3}H]DTG	(Niso et al. 2013)
	• $K_i = 0.34$ nM	Rat liver	[^{3}H]DTG	(Berardi et al. 2004)
				(Lever et al. 2014)
PD-144418	Sigma1			
	• $K_i = 0.08$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Akunne et al. 1997)
	• $K_i = 0.46$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Lever et al. 2014)
	• $K_i = 4.30$ nM	Sf9 cells	[^{3}H](+)-pentazocine	(Schmidt et al. 2016)
	Sigma2			
	• $K_i = 1{,}377$ nM	NG 108–15 cells	[^{3}H]DTG	(Akunne et al. 1997)
	• $K_i = 1{,}654$ nM	Guinea pig brain	[^{3}H]DTG	(Lever et al. 2014)
PRE-084	Sigma1			
	• $IC_{50} = 44$ nM	Guinea pig brain	[^{3}H](+)-SKF10047	(Su et al. 1991)
	• $K_i = 46$ nM	Mouse brain	[^{3}H](+)-pentazocine	(Entrena et al. 2009)
	• $K_i = 151$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Cobos et al. 2005)
	• $K_i = 53$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Garces-Ramirez et al. 2011)
	• $K_i = 9$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Brown et al. 2004)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	Sigma2			
	• $K_i = 32{,}100$ nM	Guinea pig brain	[^{3}H]DTG	(Garces-Ramirez et al. 2011)
Rimcazole	Sigma1			
	• $K_i = 406$ nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	• $K_i = 1{,}165$ nM	Guinea pig brain	[^{3}H]ne-100	(Tanaka et al. 1995)
	• $IC_{50} = 2{,}700$ nM	MDA-MB-468 cells	[^{3}H](+)-pentazocine	(Spruce et al. 2004)
	• $IC_{50} = 356$ nM	C6 rat glioma cells	[^{11}C] SA4503	(Rybczynska et al. 2008)
	• $IC_{50} = 2{,}649$ nM	Rat brain	[^{3}H](+)-SKF10047	(Roman et al. 1990)
	• $IC_{50} = 450$ nM	Guinea pig brain	[^{3}H](+)-SKF10047	(Ferris et al. 1986)
	Sigma2			
	• $K_i = 852$ nM	Rat liver	[^{3}H]DTG	(Schepmann et al. 2011)
	• $K_i = 571$ nM	RT-4 cells	[^{3}H]DTG	(Schepmann et al. 2011)
S1RA	Sigma1			
	• $K_i = 30$ nM	Mouse brain	[^{3}H](+)-pentazocine	(Nieto et al. 2012)
	• $K_i = 24$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Romero et al. 2012)
	• $K_i = 17$ nM	Not indicated	Performed by CEREP	(Romero et al. 2012)
	Sigma2			
	• $K_i = 9{,}300$ nM	Not indicated	Performed by CEREP	(Romero et al. 2012)
SA4503	Sigma1			
	• $K_i = 0.012$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Berardi et al. 2001)
	• $K_i = 4.63$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Lever et al. 2006)
	• $IC_{50} = 7$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Lever et al. 2006)
	• $IC_{50} = 17$ nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Matsuno et al. 1996a, b)
	Sigma2			
	• $K_i = 63$ nM	Guinea pig brain	[^{3}H]DTG	(Lever et al. 2006)
	• $K_i = 77$ nM	Rat liver	[^{3}H]DTG	(Berardi et al. 2001)

(continued)

Table 2 (continued)

Compound name	Binding affinity (K_i, K_d, IC_{50})	Cell lines/ tissue tested	Radioligand used	Reference
	• IC_{50} = 71 nM	Guinea pig brain	[^{3}H]DTG	(Lever et al. 2006)
	• IC_{50} = 1,784 nM	Guinea pig brain	[^{3}H]DTG	(Matsuno et al. 1996a, b)
SH-344	Sigma1			
	• K_i = 2.5 nM	Rat liver	[^{3}H](+)-pentazocine	(Hellewell et al. 1994)
	• K_i = 2.8 nM	C6 rat glioma cells	[^{3}H](+)-pentazocine	(Vilner et al. 1995a, b)
	Sigma2			
	• K_i = 43 nM	Rat liver	[^{3}H]DTG	(Hellewell et al. 1994)
Siramesine	Sigma1			
	• K_i = 10 nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Niso et al. 2013)
	• IC_{50} = 17 nM	Rat brain	[^{3}H](+)-pentazocine	(Perregaard et al. 1995)
	Sigma2			
	• K_i = 13 nM	Rat liver	[^{3}H]DTG	(Niso et al. 2013)
	• IC_{50} = 0.12 nM	Rat brain	[^{3}H]DTG	(Perregaard et al. 1995)
SR31747A	Sigma1			
	• K_i = 1 nM	MDA-MB-468 cells	[^{3}H](+)-pentazocine	(Maher et al., unpublished data)
	• K_i = 3 nM	Guinea pig brain	[^{3}H](+)-pentazocine	(Laggner et al. 2005)
	• K_d = 0.15 nM	Yeast membrane	[^{3}H] SR31747A	(Jbilo et al. 1997)

2 Sigma1 and *SIGMAR1* Expression in Tumors

Elevated expression levels of a protein or of the mRNA transcripts encoding the protein are often used to justify its relevance in cancer. In this section, we will review the literature describing the expression of *SIGMAR1* mRNA transcripts and Sigma1 protein by immunohistochemistry (IHC) and radioligand binding in tumors.

2.1 Sigma1 Protein Expression in Tumors by Immunohistochemistry

Compared to other cancer-associated proteins, there are relatively few published reports of Sigma1 immunostaining in tumors. These data are summarized in Table 3.

In one of the first reports, Casellas and colleagues performed Sigma1 IHC staining analysis of tumors from 95 breast cancer patients (Simony-Lafontaine et al. 2000). The authors found a positive correlation between Sigma1 protein and hormone receptor levels; the strongest positive correlation was with the progesterone receptor (PR) ($P = 0.01$). Interestingly, the *SIGMAR1* transcriptional promoter region contains a PR binding site (Seth et al. 1997). Together, these data suggest that Sigma1 expression may be regulated by steroid hormone feedback mechanisms. This was proposed as a rationale for considering Sigma1 as a marker to identify patients who may benefit from adjuvant hormone therapy (Simony-Lafontaine et al. 2000).

In this study, Sigma1 protein levels showed no significant positive correlation with tumor size, histological grade, nodal status, tumor proliferation (by Ki67), patient age, or whether the patients were pre- or post-menopausal. However, the absence of detectable Sigma1 was most frequently observed in tumors from post-menopausal women (Simony-Lafontaine et al. 2000).

The authors used a mouse monoclonal anti-Sigma1 antibody raised against full-length Sigma1 that was generated by the authors [first described in (Jbilo et al. 1997)]. The epitope(s) on Sigma1 was (were) not identified (Jbilo et al. 1997). An antigen retrieval step in the IHC protocol was not reported. These are important technical considerations, because depending upon the epitope against which the antibody was generated an antigen retrieval step may be needed to reveal the epitope(s) masked by formalin/formaldehyde cross-linking of the protein of interest (Leong and Leong 2007; Marchio et al. 2011). This is noteworthy because the published IHC analyses of Sigma1 in tumors, described here and below, were based on the use of different anti-Sigma1 antibodies (some without indicated epitopes) and possible variability in tissue processing and immunostaining specificity. Therefore, some of the differences in the conclusions drawn from these studies could be attributed to technical factors and may not necessarily reflect biological or clinical differences.

In a subsequent study Wang et al. performed Sigma1 IHC staining analysis of 109 tissue specimens comprising malignant breast tumors, benign breast tumors, and normal breast tissue from 58 breast cancer patients. The authors reported that Sigma1 protein was present in 60% of invasive cancers, 41% of in situ cancers, 75% of ductal hyperplasias, and 33% of normal breast tissue (Wang et al. 2004). They reported no statistically significant correlation between Sigma1 protein levels and histological grade, nodal status, and patient age. In contrast to the study by Simony-Lafontaine et al., Wang et al. found no statistically significant correlation between Sigma1 levels and estrogen receptor or progesterone receptor status. This difference may be attributable to technical factors such as different antibodies and IHC procedures as

Table 3 Immunohistochemical Analysis of Sigma1 Protein in Tumors

Reference	Cancer	Results and conclusions	Antibody used	Antigen retrieval
(Simony-Lafontaine et al. 2000)	*Breast Adenocarcinoma* (tumors from 95 breast cancer patients)	No significant correlation with tumor size, histological grade, nodal status, tumor proliferation (by Ki67), patient age, or whether the patients were pre- or post-menopausal. Significant correlation with progesterone receptor status	Mouse monoclonal anti-Sigma1 antibody against full-length Sigma1 that was generated by the authors [described in (Jbilo et al. 1997)]	An antigen retrieval step in the IHC protocol was not indicated
(Wang et al. 2004)	*Breast Adenocarcinoma* (malignant breast tumors, benign breast tumors, normal breast tissue from 58 breast cancer patients)	No significant correlation between Sigma1 protein levels and histological grade, nodal status, and patient age; no statistically significant correlation between Sigma1 levels and estrogen receptor or progesterone receptor	Goat anti-Sigma1 polyclonal antibody raised against unspecified epitope (Sigma1 L-20 antibody, Santa Cruz biotechnology, Inc.). The specificity of this antibody for Sigma1 was not confirmed	Antigen retrieval prior to IHC was performed in this study
(Xu et al. 2012)	*Esophageal Squamous Cell Carcinoma* (18 low-grade dysplasia, 8 high-grade dysplasia, 18 carcinoma, 12 non-cancerous epithelium from 18 patients)	Significant correlation with pathologic TNM classification; positive correlation with tumor size; Sigma1-positive rates generally lower in normal epithelia than in ESCC tissue	Rabbit anti-Sigma1 polyclonal antibody (Abgent). Antibody generated against a synthetic peptide, residues 47–81 of human Sigma1. The specificity of this antibody for Sigma1 was not confirmed	Antigen retrieval prior to IHC was performed in this study
(Xu et al. 2014)	*Hilar Cholangio-carcinoma (HC)* (92 HC and paired normal bile duct epithelial tissue)	Significant correlation between the percentage of tumors positive for Sigma1 immunostaining and tumor differentiation (increase in poorly differentiated tumors), lymph node metastasis, disease stage; no correlation between Sigma1 staining and tumor size or brain metastasis	Rabbit polyclonal antibody raised against an unspecified synthetic peptide derived from the C-terminal region of rat Sigma1 (ab53852; Abcam). The specificity of this antibody for Sigma1 was not confirmed	An antigen retrieval step in the IHC protocol was not indicated

well as different patient populations. However, both studies report heterogeneous expression of Sigma1 in invasive breast tumors.

The authors concluded that Sigma1 protein levels did not correlate with patient survival and were not predictive of adjuvant chemotherapy efficacy in this study. They included the caveat that their study should be considered exploratory and that it was not performed to formally evaluate prognostic value, adding that their conclusion regarding lack of statistically significant correlation may have been due to an underpowered study (Wang et al. 2004).

Xu et al. reported that Sigma1 is upregulated in esophageal squamous cell carcinoma (ESCC) and that the upregulation correlates with the pathologic tumor, node, metastasis (TNM) classification (Xu et al. 2012). The authors describe both cytoplasmic and nuclear Sigma1 immunostaining. They also report that nuclear Sigma1 has a stronger positive correlation with TNM classification and lymph node metastasis and suggest that nuclear Sigma1 may contribute to malignant progression of ESCC tumors. This group also found a significant positive correlation between Sigma1 expression and tumor size. They evaluated normal epithelium of the esophagus and compared to ESCC tissue and found that Sigma1-positive immunostaining in non-cancerous epithelium was inconsistent (33.3%, 4 of 12); however, Sigma1-positive rates were generally lower than in ESCC tissue, wherein a pattern of increasing rates of positive Sigma1 staining was observed with low-grade dysplasia (22.2%, 4 of 18) to high-grade dysplasia (61%, 11 of 18). A significant difference was observed, with 35% for low-grade dysplasia versus 60% for ESCC.

The presence or absence of Sigma1 failed to show correlation with ESCC patient survival rates; patients with high Sigma1 immunostaining had 5-year overall survival rates of 29.7% compared to 37.5% for patients with low Sigma1 immunostaining. The authors propose that Sigma1 contributes to ESCC pathogenesis and could be regarded as a novel biomarker in the prognosis of ESCC; however, they also state that their study should be regarded as exploratory (Xu et al. 2012).

Xu et al. evaluated the levels of Sigma1 in hilar cholangiocarcinoma (HC) tumors, a hepatobiliary cancer that occurs at the confluence of the right and left hepatic ducts (Xu et al. 2014). The authors performed Sigma1 IHC analysis of tissue microarrays (TMA) containing 92 HC and paired non-cancerous bile duct epithelial tissue. They report overexpression of Sigma1 in 46.7% of the HC tumors. Under their experimental conditions 53% of HC tumors presented low or no Sigma1 immunostaining, and all non-cancerous bile duct epithelial cells presented no or weak Sigma1 immunostaining. The authors report primarily cytoplasmic Sigma1 immunostaining (Xu et al. 2014).

This study found a significant positive correlation between the percentage of tumors that were positive for Sigma1 immunostaining and tumor differentiation (increased in poorly differentiated tumors), lymph node metastasis, and disease stage. However, they found no significant correlation between Sigma1 staining and tumor size or brain metastasis (Xu et al. 2014). The frequency of Sigma1 immunostaining significantly increased with disease stage, with 32.4% Sigma1 positive at TNM classification stage I/II and 56.4% at stage III/IV. They also report

that Sigma1 levels positively correlated with disease progression, poor prognosis, earlier recurrence, and diminished overall survival. HC patients with high intensity Sigma1 immunostaining presented significantly earlier recurrence (15 versus 30 months) and significantly shorter median survival duration (15 versus 42 months) compared to patients with low or no Sigma1 immunostaining. The authors report that tumor invasion, lymph node metastases, and Sigma1 immunostain intensity were independent predictive factors for tumor recurrence (Xu et al. 2014).

2.2 Sigma1 Protein Levels in Tumors Determined by Radioligand Binding

One of the first reports of the presence of sigma receptors in tumors (at the time identified as sigma binding sites) was published by Coscia and colleagues (Bowen et al. 1988; Thomas et al. 1990). The authors evaluated the density of sigma binding sites as well as opioid receptors in human brain tumors and neuroblastoma and glioma cell lines. Sigma receptor binding was performed with [^{3}H]1,3-di-o-tolylguanidine ([^{3}H]DTG) in the absence or presence of haloperidol to differentiate specific and non-specific binding. Elevated sigma binding site density was detected in 15 of 16 tumors. All brain tumor specimens were obtained from patients immediately after surgical resection. [^{3}H]DTG bound membrane preparations of meningioma with a K_d of 37–57 nM and B_{max} 683–1,260 fmol/mg protein compared to [^{3}H]DTG binding of temporal cortex tissue preps with a K_d of 60 nM and B_{max} 249 $\pm$ 105 fmol/mg protein (mean $\pm$ SE). A brain metastasis from adenocarcinoma of the lung expressed five- to tenfold greater [^{3}H]DTG than other brain tumors (Thomas et al. 1990). A caveat of this study is that haloperidol has affinity for both Sigma1 and Sigma2 binding sites; therefore, these conditions would not distinguish these two binding sites (Thomas et al. 1990).

Subsequently, this group reported increased sigma binding site density in non-neural tumors, including surgical specimens of renal and colorectal carcinoma and sarcoma (Bem et al. 1991). The freshly resected set of 9 tumors comprised 2 colon carcinoma liver metastases, 6 renal carcinomas, and 1 sarcoma metastasis. The tumors were compared to normal renal tissue and colon mucosa specimens excised from tissue adjacent to primary tumors as well as from tissue obtained during necropsy of non-cancer patients (Bem et al. 1991).

2.3 *SIGMAR1* Transcript Levels in Tumors

Systematic analyses of *SIGMAR1* gene expression, genome wide association studies, mutational analyses, or epigenetic analyses have not been reported. However, several comprehensive and well-annotated cancer focused gene expression databases are now available. These include The Cancer Genome Atlas (TCGA) (Weinstein et al. 2013) and Oncomine [https://www.oncomine.org/, first described by Chinayan and colleagues (Rhodes et al. 2004)]. These databases are a rich source

of information regarding the genomic, genetic, and epigenetic status of *SIGMAR1* in cancer that awaits data mining, analysis, and reporting. Recently, Crottès et al. reported elevated levels of *SIGMAR1* transcripts in colorectal cancers (CRC), acute myeloid leukemia (AML), and chronic myeloid leukemia (CML) compared to their paired normal tissue based on their analysis of the Oncomine database (Crottes et al. 2016).

A few focused studies have used reverse transcriptase polymerase chain reaction (RT-PCR) based approaches to quantify *SIGMAR1* transcript levels in cancer tissue specimens. In one of the earliest such studies, Wang et al. evaluated the relative *SIGMAR1* transcript levels in 14 breast cancer specimens by quantitative real-time RT-PCR (qRT-PCR). They found that 9 of 14 (64%) of the samples had elevated *SIGMAR1* (ratio of *SIGMAR1* in cancer tissue to a pool of normal breast tissue). The ratio of *SIGMAR1* in cancer versus normal tissue ranged from 2 to 37, with a median ratio of 4 (2.85 at 25% and 17.75 at 75%). However, in 5 of 14 (36%) breast cancer samples the authors found less *SIGMAR1* compared to the reference pool of normal breast tissue, with ratios ranging from 0.8 to 0.02, with a median ratio of 0.11 (0.025 at 25% and 0.51 at 75%) (Wang et al. 2004).

Although not specifically addressed in a study of gene expression in malignant melanoma and benign melanocytic lesions by Talantov et al., a closer review of the data in this publication revealed that some malignant melanomas express extremely high levels of *SIGMAR1* transcripts compared to benign tissue controls (Talantov et al. 2005). The *SIGMAR1* gene transcript data can be found at the NCBI GEO Profile for this study (accession number GSE3189).

Skrzycki and Czeczot used semi-quantitative RT-PCR to evaluate *SIGMAR1* transcript levels in tumors from 30 CRC patients, 18 with primary CRC and 12 with liver metastatic CRC. Using this method, the authors concluded that relative *SIGMAR1* transcript levels are highest in stage III CRC based on the TNM staging system (Skrzycki and Czeczot 2013). This study also reported significantly decreased levels of *SIGMAR1* transcripts in older CRC patients. The authors conclude that increased *SIGMAR1* correlates with CRC stage and metastasis and decreases with patient age (Skrzycki and Czeczot 2013).

Analysis of *SIGMAR1* in patient tumors in the Oncomine and The Cancer Genome Atlas (TCGA) databases and survey of the literature reveals that Sigma1 is not uniformly upregulated in tumors. Interestingly, even among clinical subtypes and individual patients of each cancer, there is variability in the magnitude of enrichment of Sigma1. The significance of this variability in expression is unclear.

3 Sigma1 and *SIGMAR1* Expression in Cancer Cell Lines

3.1 Sigma1 Protein in Cancer Cell Lines Determined by Immunoblot

A number of groups have reported Sigma1 protein expression in cancer cell lines by immunoblot; a few are listed here (Vilner et al. 1995b; John et al. 1995b; Spruce et al. 2004; Aydar et al. 2006; Wang et al. 2004; Kim et al. 2010, 2012; Xu et al. 2012; Schrock et al. 2013; Thomas et al. 2017). Aydar et al. confirmed Sigma1 protein expression by immunoblot in lung (H69, H209, H510), breast (MDA-MB-361, MDA-MB-435, BT20 and MCF-7), and prostate (PC3, LNCaP) cancer cell lines (Aydar et al. 2006). Wang et al. performed immunoblots to confirm Sigma1 protein expression in MDA-MB-231, MDA-MB-361, MDA-MB-435, MCF-7, and BT20 breast cancer cell lines (Wang et al. 2004). In their hands, T47D cells did not express Sigma1. This is inconsistent with other reports (Kim et al. 2012; Schrock et al. 2013; Vilner et al. 1995b). MCF-7 cells were initially reported to be Sigma1 negative (Vilner et al. 1995b); however, a number of studies demonstrate that MCF-7 cells express Sigma1 and *SIGMAR1* and are responsive to Sigma1 ligands (Vilner et al. 1995b; John et al. 1995b; Spruce et al. 2004; Aydar et al. 2006; Wang et al. 2004; Kim et al. 2012; Schrock et al. 2013). Xu et al. reported Sigma1 protein expression in human esophageal squamous cell carcinoma (ESCC) cell lines KYSE150, KYSE180, and EC109 (Xu et al. 2012). Kim and colleagues confirmed Sigma1 protein expression by immunoblot in prostate cancer (LAPC4, LNCaP, C4-2, 22Rv1, VCaP, PC3, DU145), breast cancer (T47D, MCF-7, MDA-MB-231, MDA-MB-468, SKBR3, BT474), pancreas (Panc1), liver cancer (HepG2), and neuroblastoma (SK-N-BE(2)C) cell lines (Kim et al. 2010, 2012; Schrock et al. 2013; Thomas et al. 2017). To date, no clearly Sigma1-negative cancer cell line has been identified.

3.2 Sigma1 Binding Sites in Cancer Cell Lines Evaluated by Radioligand Binding

Most radioligand binding studies to detect and quantify Sigma1 in cancer cell lines were performed with the following three radioligands: [^{3}H](+)-pentazocine, [^{3}H](+)-SKF10047, and [^{3}H]DTG (Table 2). The pharmacological selectivity and specificity of the first two prototypic Sigma1 ligands was confirmed by a study with *SIGMAR1* homozygous knockout mice (Langa et al. 2003). In this study, [^{3}H](+)-pentazocine did not bind to brain membrane preparations from *SIGMAR1*$^{-/-}$ mice, and (+)-SKF10047 stimulation of locomotor activity was not observed in these mice (Langa et al. 2003).

High levels of Sigma1 have been quantified in a number of human and rodent cancer cell lines by radioligand binding saturation assay. These assays have been performed and Sigma1 was detected on extracted cell membrane preparations from cell lines of prostate cancer (Vilner et al. 1995b), breast cancer (Crawford and

Bowen 2002; Vilner et al. 1995b; Spruce et al. 2004; Schrock et al. 2013), colon cancer (Bem et al. 1991), melanoma (Vilner et al. 1995b), small- and non-small-cell lung carcinoma (Maneckjee and Minna 1992; John et al. 1995a; Moody et al. 2000; Vilner et al. 1995b), renal cancer (Bem et al. 1991), bladder cancer (Schepmann et al. 2011), brain tumors (Thomas et al. 1990), glioblastoma (Vilner et al. 1995b), neuroblastoma (Ryan-Moro et al. 1996; Vilner et al. 1995b), multiple myeloma (Brune et al. 2012), and sarcoma (Bem et al. 1991).

Sigma1 has been detected by radioligand binding on a number of rodent cancer cell lines as well, including C6 rat glioma (Vilner et al. 1995b), N1E-115 rat neuroblastoma (Vilner et al. 1995b), NG108-15 rat neuroblastoma x glioma hybrid (Vilner et al. 1995b), and TRAMP (transgenic adenocarcinoma mouse prostate) cells (Colabufo et al. 2008).

3.3 Accumulation of Sigma1 Radioligands in Xenografted Tumors In Vivo

Bowen and colleagues performed Sigma1 ligand biodistribution studies in nude mice xenografted with a human prostate cancer cell line (DU145). They demonstrated that radioiodinated benzamides with affinity for Sigma1 appeared to be retained in tumors compared to normal tissues. 4-[^{125}I]-PAB, [^{125}I]-PIMBA, 2-[^{125}I]-BP had tumor/blood ratios of 14, 70, and 41 at 6 h post-injection, respectively. 4-[^{125}I]PAB, [^{125}I]-PIMBA, 2-[^{125}I]-BP had tumor/muscle ratios of 57, 70, and 28 at 6 h post-injection, respectively. 2-[^{125}I]-BP had tumor/blood and tumor/muscle ratios of 35 for both at 24 h post-injection. These data suggest that Sigma1 ligands may preferentially accumulate in tumors compared to other normal tissue (John et al. 1999).

Moody et al. performed a similar biodistribution experiment with [^{125}I]-*N*-(2-(piperidino)ethyl)-2-iodobenazmide (2-IBP) in mice xenografted with NCI-N417 non-small-cell lung cancer (NSCLC) cells. In this study as well, the Sigma1 ligand was present in higher concentrations in tumors compared to normal tissue (Moody et al. 2000).

Xie et al. synthesized an ^{18}F labeled piperidine compound, 8-(4-(2-[^{18}F] fluoroethoxy)benzyl)-1,4-dioxa-8-azaspiro[4.5]decane ([^{18}F]5a), with high affinity for Sigma1 ($K_i = 5.4$ nM). The authors demonstrate specific intracellular Sigma1 binding by [^{18}F]5a in vitro in four cancer cell lines, PC3 and DU145 (prostate adenocarcinoma), MCF-7 (breast adenocarcinoma), and A375 (melanoma). Specificity of [^{18}F]5a binding to Sigma1 was confirmed with cold blocking ligands haloperidol, SA4503, and fluspidine in cellular uptake assays with all four human cancer cell lines. Consistent with the radioligand binding data, these cell lines have been reported to express different levels of *SIGMAR1* transcripts and Sigma1 by immunoblot. By autoradiography and positron emission tomography (PET) imaging, the authors demonstrate accumulation of high levels of [^{18}F]5a in subcutaneously xenografted tumors of the above cell lines in mice. Accumulation was highest in PC3 tumors > A431 > A375 > DU145. The accumulation of the [^{18}F]5a

radiotracer in PC3 and A431 xenografted tumors was significantly decreased by co-administration with haloperidol, suggesting Sigma-selective binding of this radiotracer (Xie et al. 2015).

SA4503 (1-(3,4-dimethoxyphenethyl)-4-(3-phenylpropyl)piperazine dihydrochloride) is a high affinity Sigma1 selective small molecule ligand with negligible affinity for at least 36 other receptors and ion channels (Matsuno et al. 1996b).

A number of reports suggest that SA4503 may be a promising Sigma1 targeted tumor radiotracer (Kawamura et al. 2005; Rybczynska et al. 2009; van Waarde et al. 2004, 2006; Ye et al. 2016). Proposed advantages of [^{11}C]SA4503 are its improved selectivity for tumor cells in inflamed tissue compared to ^{18}F-fluorodeoxyglucose (^{18}F-FDG) (van Waarde et al. 2006) as well as its high tumor uptake and retention (van Waarde et al. 2004, 2006). Ramakrishnan et al. found twofold higher levels of [^{11}C]SA4503 accumulation in spontaneous pituitary tumors compared to normal pituitary tissue (Ramakrishnan et al. 2013). Van Waarde et al. evaluated [^{11}C]SA4503 as a PET ligand in rodent models. The authors reported that 1 h post-injection [^{11}C]SA4503 accumulated in C6 tumors at a tumor-to-plasma ratio of 13.4 and a tumor-to-muscle ratio of 5.0 (van Waarde et al. 2004). Kawamura et al. reported that [^{11}C]SA4503 accumulated in AH109A hepatoma xenografted tumors in rats. Uptake in this cell line decreased by carrier-loading and pre-treatment with haloperidol ([^{11}C]SA4503, 41% and 22%, respectively, at 30 min after injection), in support of Sigma1 specific binding and accumulation (Kawamura et al. 2005).

Together, these and other studies not reviewed here suggest that radiolabeled Sigma1 ligands preferentially accumulate in tumors and are promising radiotracers for tumor imaging in vivo. Interestingly, this is true even when comparing cancer cells with normal tissues that express high levels of Sigma1 protein, suggesting that Sigma1 may exist in a distinct binding conformation in cancer cells.

3.4 *SIGMAR1* Transcript Levels in Cancer Cell Lines

The availability of well-curated and publically available databases such as Cell Miner and the Cancer Cell Line Encyclopedia (CCLE), which contain the full gene expression profile of over 1,000 cancer cell lines, provides valuable reference data sets for gene expression studies. We evaluated *SIGMAR1* mRNA transcript expression levels in 1,036 cancer cell lines in the CCLE (Fig. 1). Our analysis of these databases and survey of the literature highlights that *SIGMAR1* is not uniformly upregulated in tumors and in cancer cell lines. Interestingly, even among clinical subtypes and individual patients of each cancer, there is variability in the levels of Sigma1 and *SIGMAR1* transcripts. This is reflected in the 1,036 cancer cell lines representing >20 cancers in the CCLE (Fig. 1). The significance of this variability in expression is unclear but may reflect the context-dependent functions of Sigma1, even within a cancer type.

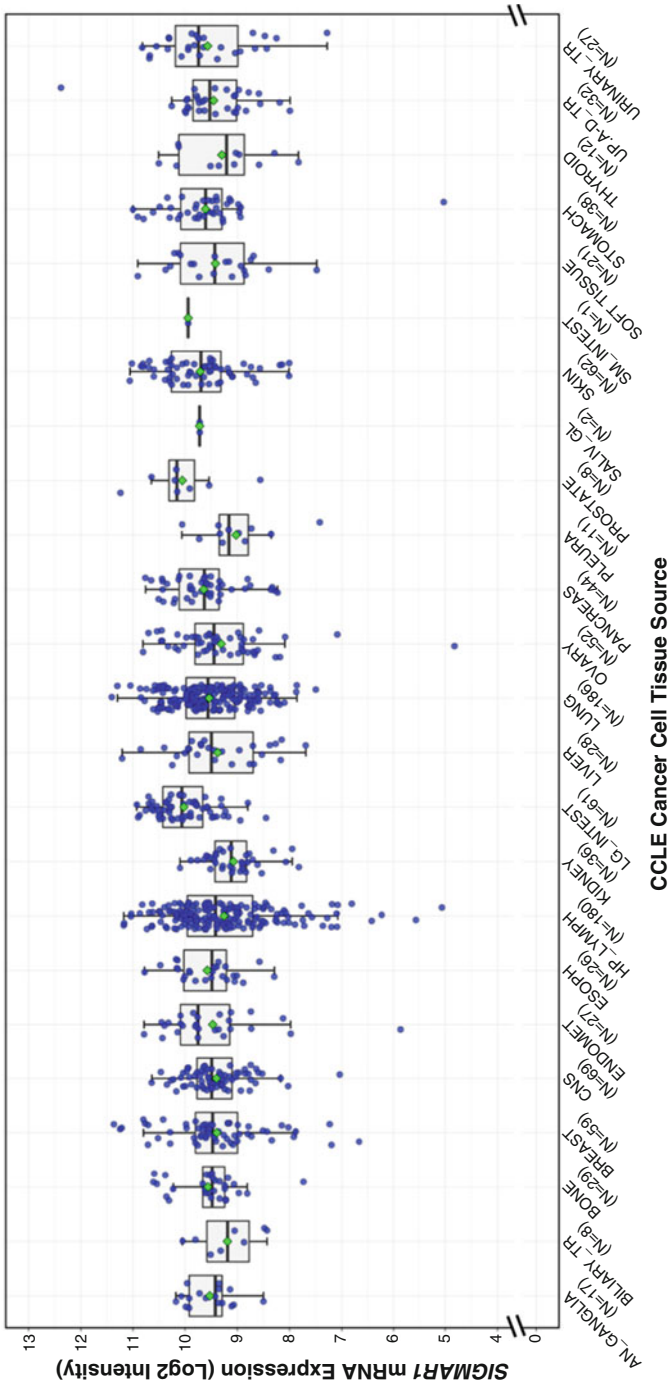

Fig. 1 *SIGMAR1* transcript levels in 1,036 cancer cell lines. Levels of *SIGMAR1* mRNA transcripts expressed as Log2 intensity values (*Y*-axis) in 1,036 cancer cell lines from the Cancer Cell Line Encyclopedia (CCLE). Tissue of origin of the cancer cell lines indicated on the *X*-axis. N = number of cell lines in each cancer type. *Blue data* points in scatter plot represent individual cell lines. Box and whisker plots show median *SIGMAR1* Log2 intensity ± standard deviation. *Green diamond* indicates median

4 Cancer Pharmacology of Sigma1 Modulators

4.1 Sigma1 Ligands: Putative Agonists and Antagonists

Despite compelling evidence that Sigma1 is not a traditional receptor, small molecule compounds with affinity for Sigma1 continue to be described as agonists and antagonists. They were originally classified on the basis of rodent behavior assays. The synthetic Sigma1 ligands di-o-tolylguanidine (DTG) and BD1052 exacerbated cocaine-induced convulsions and locomotor activity and were classified as agonists (Matsumoto et al. 2001). In contrast, other synthetic Sigma1 ligands BD1008, BD1047, BD1063, and LR172 were defined as antagonists because they attenuated cocaine-induced convulsions, abnormal hyper-locomotor activity, and lethality in mice (McCracken et al. 1999). Consistent with pharmacological antagonists, when administered alone the Sigma1 putative antagonists produced no reported changes in behavior (Matsumoto et al. 2001).

A rodent model of memory impairment was also used to classify Sigma1 compounds as agonists and antagonists. Maurice and colleagues demonstrated that Sigma1 putative agonists (+)-pentazocine, PRE-084, and SA4503 had anti-amnesic effects in a beta-amyloid-related peptide-induced memory impairment behavior assay. Neurosteroids with affinity for Sigma1 including pregnenolone, dehydroepiandrosterone, and their sulfate esters also produced a neuroprotective effect, which was interpreted as Sigma1 agonism. Progesterone and haloperidol blocked these neuroprotective effects and were thus classified as Sigma1 antagonists in this assay. Importantly, although they blocked the beneficial effects of the Sigma1 agonists in attenuating memory impairment, these Sigma1 antagonists, when administered alone, had no effect on (i.e., did not worsen or accelerate or ameliorate) 25–35 peptide-induced symptoms (Maurice et al. 1998). A number of related studies are reviewed by Maurice and Goguadze in this volume (*Sigma-1 (σ_1) Receptor in Memory and Neurodegenerative Diseases*).

In experimental models of cancer, inhibition of cancer cell proliferation and survival are considered measures of Sigma1 inhibition (putative antagonism). Spruce et al. and Colabufo et al. were among the first to propose that Sigma1 putative antagonists/inhibitors but not agonists/activators elicit antiproliferative and cytotoxic effects on cancer cells (Spruce et al. 2004; Colabufo et al. 2004). In these seminal studies, Sigma1 antagonism/inhibition, as originally defined on the basis of behavioral endpoints, generally correlated with inhibition of cancer cell proliferation and growth, and in some cases induction of apoptosis (Colabufo et al. 2004; Spruce et al. 2004). However, this does not strictly apply. For instance, although the putative agonists/activators PRE-084 and (+)-SKF10047 do not alter cell proliferation or survival in most published studies, some putative agonists/activators such as 4-IBP [*N*-(*N*-benzylpiperidin-4-yl)-4-iodobenzamide] have been reported to have antiproliferative properties on their own as well as the ability to sensitize cancer cells to proapoptotic and proautophagic drugs (Megalizzi et al. 2009, 2007).

To further complicate attempts at classification, most putative sigma ligands have affinity for both the Sigma1 and Sigma2 subtypes, albeit with broad differences in subtype binding affinity (Table 2). It has been proposed that the antiproliferative and proapoptotic activities of Sigma1 ligands may involve Sigma1 antagonism/inhibition combined with Sigma2 putative agonism (Zeng et al. 2014). However, the identity of Sigma2 is controversial (Pati et al. 2017; Abate et al. 2015) and the definition of Sigma2 agonism is also unclear.

If, based on the above, the physiological role of Sigma1 in cancer cell and tumor biology is to promote growth and survival, then what does it mean to activate or inhibit Sigma1? How can this be measured? To date, there is no established molecular or biochemical mechanism of action that can clearly define Sigma1 agonist/activator and antagonist/inhibitor activity. In contrast to GTPγS for G protein-coupled receptors (GPCR), kinase activity for receptor tyrosine kinases (RTKs), and ATP binding for heat shock protein 90 (HSP90), there are no established proximal signaling or enzymatic activities clearly attributable to Sigma1. A standard biochemical assay for defining compounds as Sigma1 agonists/activators and antagonists/inhibitors remains to be established.

4.2 Prototypic Small Molecule Ligands: Effects In Vitro and In Vivo

Despite the aforementioned uncertainty regarding the classification of Sigma1 ligands, much of our understanding of Sigma1 biology and pharmacology comes from studies with synthetic small molecule compounds (i.e., ligands). Compounds with affinity for Sigma1 have been reported to influence cell survival, apoptosis, cell proliferation, growth, cell adhesion, motility, migration, cell cycle progression, lipid homeostasis, and protein homeostasis pathways. In the absence of a coherent, unifying explanation for how Sigma1 pharmacology regulates these pathways and processes, thereby producing what appears to be the wide range of therapeutic opportunities, we have selected a number of prototypic Sigma1 ligands and provide a compound-centric survey of the literature to describe how they have been used to implicate Sigma1 in these cellular processes. In this section, we will review and analyze the reported properties and activities of a selected set of relatively widely published prototypic Sigma1 ligands.

4.2.1 (+)-SKF10047

Also known as (+)-*N*-allylnormetazocine, (+)-SKF10047 is a prototypic Sigma1 ligand and putative agonist/activator [see above and (Maurice et al. 1994; Hayashi and Su 2001)]. The Sigma1 selectivity of (+)-SKF10047 was confirmed by the absence of binding and activity in *SIGMAR1* knockout (KO) mice (Langa et al. 2003). Spruce et al. were among the first to delineate that putative Sigma1 antagonists/inhibitors, but not agonists/activators, inhibit tumor growth and survival

both in vitro and in vivo. They showed that some putative Sigma1 antagonists/inhibitors elicit caspase-mediated apoptosis, and that agonists/activators including (+)-SKF10047 and (+)-pentazocine block or attenuate this effect (Spruce et al. 2004).

In some cases, putative agonists/activators promote cancer cell proliferation and tumor growth. For example, in the same publication mentioned above, Spruce et al. show that (+)-SKF10047 and (+)-pentazocine both promoted in vitro proliferation of the MCF-7 breast cancer cell line, suggesting that some cancer cells can respond to agonistic signals that promote cell proliferation and survival (Spruce et al. 2004). In a later study, Happy et al. reported that (+)-SKF10047 treatment alone appeared to increase proliferation of MCF-7 and MDA-MB-231 cells (Happy et al. 2015). Consistent with the study by Spruce et al., Happy et al. reported that (+)-SKF10047 blocked the antiproliferative and proapoptotic effects of rimcazole in a panel of breast cancer cell lines (Happy et al. 2015).

Using the same approach as Happy et al., Saune and colleagues recently reported that treatment of DU145, LNCaP, and PC3 prostate cancer cell lines with (+)-SKF10047 or overexpression of recombinant Sigma1 prevented tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis (Das et al. 2016). The authors proposed that higher levels of active Sigma1 render prostate cancer cells resistant to TRAIL treatment. RNAi knockdown of Sigma1 sensitized TRAIL resistant T47D, MDA-MB-157, and MDA-MB-231 breast cancer cell lines to the antiproliferative and proapoptotic effects of ectopically expressed, adenoviral vector transduced TRAIL (Das et al. 2016).

In contrast, Aydar et al. reported that (+)-SKF10047 treatment significantly inhibited cell adhesion but did not affect proliferation or migration of MDA-MB-231 and MDA-MB-468 breast cancer cell lines (Aydar et al. 2016). The authors propose that Sigma1 activation alters cell adhesion through interaction with the neonatal Nav1.5 (nNav1.5) ion channel (Aydar et al. 2016). They propose that because combining Sigma1 knockdown or (+)-SKF10047 with an nNav1.5 activity blocking polyclonal antibody (NESOpAb) had similar effects as each treatment alone, cell adhesion may be mediated through a common mechanism involving Sigma1 interaction with nNav1.5 (Aydar et al. 2016). This group also reported that (+)-SKF10047 (albeit at 100 μM) inhibited MCF-7 cell adhesion by 41% and inhibited MDA-MB-231 cell adhesion by 57%. RNAi knockdown of Sigma1 in MCF-7 and MDA-MB-231 cells also resulted in 42% and 29.76% inhibition of cell adhesion, respectively (Aydar et al. 2006). Although interesting, these observations are inconsistent with a definition of (+)-SKF10047 as an agonist/activator. Nevertheless, these data were used as evidence to suggest that Sigma1 may play a role in cancer cell metastasis (Aydar et al. 2006).

Aydar and colleagues have proposed that Sigma1 also alters cell adhesion by regulating the actions of β-integrin (Palmer et al. 2007; Aydar et al. 2006). The authors of these studies postulated that RNAi knockdown (KD) of Sigma1 and (+)-SKF10047 treatment produce effects consistent with β-integrin blockade. Although the mechanisms by which (+)-SKF10047 elicits these effects were not determined, (+)-SKF10047 treatment resulted in dissociation of Sigma1 from lipid rafts and

decreased Sigma1-β-integrin association in lipid raft fractions (Palmer et al. 2007). In this study as well, the correlation between Sigma1 KD and (+)-SKF10047 treatment is inconsistent with a definition of (+)-SKF10047 as an agonist/activator. However, this suggests that Sigma1 can contribute to cholesterol content of the surrounding lipid bilayer and possibly associated proteins, such as integrins and ion channels (Palmer et al. 2007; Aydar et al. 2002, 2004; Balasuriya et al. 2014).

Disruption of cholesterols in lipid rafts alters the functionality and composition of the signaling complexes present in these organizing and stabilizing structures (Jacobson et al. 2007; Simons and Toomre 2000). Palmer et al. have proposed that Sigma1 contains two cholesterol-binding domains (CBD) that have peripheral benzodiazepine receptor and the HIV envelope glycoprotein-like CBD motifs (Palmer et al. 2007). These CBDs are adjacent to the Sigma1 ligand-binding site (Palmer et al. 2007; Schmidt et al. 2016). The authors proposed that Sigma1 contributes to lipid raft modeling and showed that Sigma1 binding to cholesterols was inhibited by (+)-SKF10047 binding to Sigma1 (Palmer et al. 2007).

4.2.2 PRE-084

Sigma1 agonists have been reported to augment the production of immune suppressive cytokines that block the host anti-tumor immune response in the tumor microenvironment. In the first report of Sigma1 ligand-mediated suppression of anti-tumor immunity, Zhu et al. showed that Sigma1 agonists/activators enhance tumor growth in part by inducing IL-10 at the tumor site (Zhu et al. 2003). They showed that the Sigma1 putative agonists/activators PRE-084 and (+)-SKF10047 induced the extracellular secretion of IL-10, TGF-β, and PGE2, while decreasing IFN-γ at the tumor site (Zhu et al. 2003). The authors demonstrated that PRE-084 promoted tumor growth in a syngeneic lung cancer model by an IL-10 dependent mechanism (Zhu et al. 2003). In the L1C2 murine alveolar cell carcinoma syngeneic tumor model, PRE-084 (20 mg/kg, i.p) and cocaine (5 mg/kg, i.p) promoted tumor growth by >2- and 3-fold, respectively. This effect was associated with induction of IL-10 at the tumor site. The tumor growth promoting effect of PRE-084 was blocked by co-administration of BD1047 (Sigma1 putative antagonist/inhibitor, thus demonstrating that these effects were Sigma1-mediated) and by an anti-IL-10 antibody (JES-2A5, thus demonstrating that IL-10 was required for the tumor growth promoting effect). Furthermore, transplantation of lymphocytes from PRE-084 treated mice transferred the immune suppressive phenotype and promoted tumor growth (Zhu et al. 2003). However, the authors did not show whether BD1047 had immunomodulatory or tumor growth inhibiting effects when administered alone. Interestingly, in contrast to tumor bearing mice, in normal mice (i.e., in the absence of tumor) treatment with Sigma1 agonists/activators did not increase the production or secretion of TGF-β (Zhu et al. 2003). Altogether, these data suggest that Sigma1 agonists/activators induce immune suppressive cytokine production by the tumor or that they promote tumor-induced cytokine production in the mouse.

4.2.3 (+)-Pentazocine

(+)-Pentazocine is a prototypic Sigma1 ligand and putative agonist/activator that is widely accepted as a reference compound for Sigma1 specific actions. [^{3}H](+)-pentazocine binding is abolished in tissue preparations from *SIGMAR1* knockout (KO) mice, confirming that it selectively binds Sigma1 (Langa et al. 2003).

Spruce and colleagues proposed that Sigma1 functions as a "brake on apoptosis" and reported that the caspase-dependent proapoptotic actions of Sigma1 antagonists were attenuated by (+)-SKF10047 and (+)-pentazocine (Spruce et al. 2004). This group also reported that rimcazole induced hypoxia inducible factor-1alpha (HIF-1α) protein levels under normoxic conditions in colorectal (HCT-116) and mammary carcinoma (MDA-MB-231) cell lines. They concluded that induction of HIF-1α contributes to cancer cell apoptosis by rimcazole (Achison et al. 2007). (+)-pentazocine blocked induction of HIF-1α by rimcazole, supporting that this is, at least in part, a Sigma1-mediated effect. (+)-pentazocine also inhibited HIF-1α induction and response by the anoxia mimetic deferoxamine mesylate (DFX), suggesting that Sigma1 opposes HIF-1α induction in response to anoxia.

Renaudo et al. reported that sigma ligand-mediated blockade of voltage-gated K + channels inhibited proliferation of small cell lung cancer (SCLC, NCI-H209, and NCIH146) and leukemic (Jurkat) cells. They found that three putative agonists/activators, (+)-pentazocine, igmesine, and DTG, all reversibly inhibited voltage-activated K+ currents, in order of descending potency. Consistent with K+ channel blockers tetraethylammonium (TEA) and 4-aminopyridin, treatment of Jurkat and SCLC cells with these sigma ligands resulted in accumulation of the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and decreased cyclin A expression and corresponding G1 cell cycle arrest (Renaudo et al. 2004). Of note, it has been reported that the IC_{50} for blockade of K+ current is 10 times higher in normal cells (Soriani et al. 1998; Lupardus et al. 2000) than in the leukemic and SCLC cell lines.

These results showing that putative Sigma1 agonists/activators can elicit cell cycle arrest and inhibit cancer cell proliferation are inconsistent with other data demonstrating the cell growth and proliferation promoting effects of Sigma1 agonists/activators (see above). It is difficult to reconcile these discrepancies. A systematic evaluation of a broad panel of Sigma1 ligands using a set of cancer cell lines should provide clarity. However, in most publications, (+)-pentazocine alone has no effect on in vitro proliferation or survival of a broad range of cancer cell lines (Labit-Labit-Le Bouteiller et al. 1998; Colabufo et al. 2004; Spruce et al. 2004; Rybczynska et al. 2008; Achison et al. 2007; Wang et al. 2004; Abate 2012; Megalizzi et al. 2012; van Waarde et al. 2015; Brust et al. 2014).

4.2.4 4-IBP

4-(*N*-benzylpiperidin-4-yl)-4-iodobenzamide (4-IBP) was originally synthesized and evaluated as a radiopharmaceutical for in vivo tumor imaging. [^{125}I]-*N*-(*N*-benzylpiperidin-4-yl)-4-iodobenzamide (4-[^{125}I]BP) binds Sigma1 with high affinity, $K_d = 26$ nM. DTG and haloperidol were shown to displace 4-[^{125}I]BP with K_i values of 4.6 and 56 nM, respectively, in MCF-7 cells (John et al. 1994, 1995b). It

was later classified as an agonist or inverse agonist based on its modulation of glutamatergic responses in hippocampal neurons (Bermack and Debonnel 2005).

Mégalizzi et al. reported that 4-IBP had weak antiproliferative effects on human glioblastoma (U373-MG) and melanoma (C32) cell lines, producing $\leq$10% inhibition of proliferation after 3 days of treatment in vitro (Megalizzi et al. 2007). Human NSCLC (A549) and prostate cancer (PC3) cells were more sensitive. However, in vitro cell migration and motility of all four cell lines were suppressed by sub-micromolar concentrations of 4-IBP using live-cell phase-contrast microscopy. In this study, inhibition of U373-MG cell motility or the organization of the actin cytoskeleton after treatment with 4-IBP was not associated with changes in intracellular [Ca^{2+}] (Megalizzi et al. 2007). This contrasts with other reports that Sigma1 ligand induced changes to cancer cell cytoskeleton occur by regulating ER Ca^{2+} efflux through Sigma1 associated IP3R (Hayashi and Su 2001).

In vivo, co-administration with 4-IBP extended survival of temozolomide treated orthotopic (brain) U373-MG glioblastoma xenograft-bearing mice, suggesting that Sigma1 ligands can potentiate the therapeutic benefit of a standard of care agent in the treatment of glioblastoma (Megalizzi et al. 2007). In an A549 metastatic NSCLC orthotopic tumor xenograft model, co-administration of 4-IBP and irinotecan significantly extended survival compared to either drug alone. Tumor analysis (i.e., tumor growth inhibition or biochemical analysis of tumors) was not reported (Megalizzi et al. 2007).

Though their rationale for evaluating these processes is unclear, the authors report that 4-IBP did not induce autophagy or UPR in U373-MG glioblastoma cells; however, 4-IBP sensitized this cell line to proapoptotic (lomustin) and proautophagic (temozolomide) compounds in vitro (Megalizzi et al. 2007).

4.2.5 Adamantane Phenylalkylamines

Riganas et al. describe a series of adamantane phenylalkylamines with affinity for Sigma1 that had antiproliferative effects in vitro on cell lines representing colon cancer (HCT-116, HCT-15), androgen independent prostate cancer (DU145, PC3), hormone-sensitive breast cancer (MCF-7), ovarian cancer (OVCAR-5), brain cancer (U-251), leukemia (HL-60), pancreatic cancer (BxPC-3), and liver cancer (SK-HEP-1). These effects were associated with cell cycle arrest and in some instances, apoptosis (Riganas et al. 2012a, b, c). A particularly interesting analogue, which they named 4a, suppressed growth of xenografted pancreatic (BxPC-3), prostate (PC3, DU145), and ovarian (OVCAR-5) tumors in SCID mice (Riganas et al. 2012a, b, c). The authors report that 4a may also have antimetastatic (measured by decreased incidence of secondary tumors) and analgesic (attenuation of paclitaxel and formalin induced pain using a previously described paw-lick assay) properties (Coderre et al. 1990; Laughlin et al. 2002; Matsumoto et al. 2006; Riganas et al. 2012a, b, c).

4.2.6 Igmesine

Soriani and colleagues have published a series of studies demonstrating the involvement of Sigma1 in ion channel activity (Balasuriya et al. 2014; Crottes et al. 2016, 2011; Gueguinou et al. 2017; Renaudo et al. 2004, 2007). A number of these studies used igmesine (Gueguinou et al. 2017; Crottes et al. 2011; Renaudo et al. 2004, 2007).

Renaudo et al. showed that three Sigma1 putative agonists/activators blocked voltage-activated K+ current amplitude in SCLC (NCI-H209, NCI-H146) and leukemic (Jurkat) cells (Renaudo et al. 2004). This effect was observed with a rank order potency of igmesine > (+)-pentazocine > DTG. Igmesine reduced Jurkat cell density, in vitro, by 23.9 and 82.8% at 10 and 30 μM, respectively, after 3 days of culture. This effect was also observed with Kv1.3 channel blockers tetraethylammonium (TEA) and 4-aminopyridin. Inhibition of cell proliferation by igmesine was associated with accumulation of total cellular levels of cyclin-dependent kinase inhibitor $p27^{Kip1}$ and a decrease in cyclin A expression. However, it is unclear whether there were increased levels of $p27^{Kip1}$ in the nucleus of these cells. The authors conclude that Sigma1 ligands can inhibit cancer cell cycle progression and thus proliferation in part through inhibition of K+ channel conductance (Renaudo et al. 2004).

Pharmacological regulation of the potassium channel Kv1.3 by igmesine appears to occur through a mechanism that does not involve changes in the cellular expression or levels of Kv1.3, as igmesine does not alter cellular Kv1.3 levels, at least in chronic lymphocytic leukemia (B-CLL) cells (Szabo et al. 2015). This is consistent with a report from Soriani and colleagues that hERG levels and surface expression are not altered by igmesine in chronic myelogenous leukemia (K562) and human embryonic kidney fibroblast (HEK293) cell lines (Crottes et al. 2011).

Igmesine has been evaluated in clinical trials for depression and diarrhea (Roze et al. 1998; Volz and Stoll 2004). The compound had acceptable safety and PK properties for the depression trial and advanced to Phase III. However, it did not reach statistically significant efficacy in the larger patient population studies in Phase III (Roze et al. 1998; Volz and Stoll 2004).

4.2.7 Haloperidol

In one of the first reports of the anti-cancer cell effects of Sigma1 ligands, Vilner, Costa, and Bowen discovered that haloperidol, reduced haloperidol, BD737, BD1008, SH344, and JL-II-147 produced morphological changes consistent with cytotoxicity in human neuroblastoma cell lines SK-N-SH and SH-SY5Y in vitro (Vilner and Bowen 1993; Vilner et al. 1995a). Additionally, a number of other neuroleptic agents with affinity for Sigma1 inhibited in vitro proliferation and survival of C6 glioma cells, albeit at high concentrations, with the following rank order potency: fluphenazine = perphenazine = haloperidol = reduced haloperidol > pimozide = spiperone >>(−)-sulpiride. At the same concentrations, neuroleptic compounds without affinity for Sigma1 lacked antiproliferative or cytotoxic properties (Vilner and Bowen 1993; Vilner et al. 1995a).

Several subsequent publications confirmed the in vitro cancer cell proliferation and cell survival inhibiting effects of haloperidol. Haloperidol and reduced haloperidol inhibited in vitro cell proliferation of MDA-MB-361, MDA-MB-435, MDA-MB-231, BT20, and MCF-7 cells (Wang et al. 2004). Haloperidol had antiproliferative and anti-migratory effects on glioblastoma cells in vitro (Rybczynska et al. 2008; Megalizzi et al. 2009). It also suppressed NCI-N417 lung carcinoma cell growth and survival in proliferation and clonogenic assays in vitro (Moody et al. 2000). Haloperidol inhibited proliferation and induced apoptosis of mouse (B16) and human (SK-MEL-28) melanoma cell lines (Nordenberg et al. 2005). Furthermore, reduced haloperidol combined with doxorubicin, vinorelbine, paclitaxel, and docetaxel produced additive cytotoxic effects in vitro (Wang et al. 2004).

In one study, haloperidol had modest in vivo tumor growth inhibiting properties in xenograft experiments. Combination of haloperidol and an EGFR inhibitor (AG1478) was reported to significantly delay tumor growth in a subcutaneous U87MG glioblastoma xenograft model. At 37 days of treatment, average xenografted tumor volume with combination treatment reportedly suppressed tumor volume to 17% of vehicle treated control mice, whereas tumors in mice treated with either AG1478 or haloperidol alone had average tumor volumes of 49% and 86% of control tumors, respectively (Li et al. 2014).

4.2.8 SR31747A

SR31747A (*N*-cyclohexyl-*N*-ethyl-3-(3-chloro-4-cyclohexylphenyl)propen-2-ylamine hydrochloride) is a high affinity ($K_i = 3$ nM) Sigma1 putative antagonist/inhibitor that was initially characterized as an immune suppressive agent (Casellas et al. 2004). In murine models of acute and chronic inflammation, SR31747A elicited a dose-related inhibition of proliferative response to mitogens – including concanavalin A, allogeneic stimulation, or phorbol myristate acetate (PMA) plus interleukin-2 (IL-2) – of mouse and human lymphocytes (Casellas et al. 1994). SR31747A modulated the production of pro- and anti-inflammatory cytokines. In SR31747A-treated mice, production of the anti-inflammatory cytokine IL-10 was induced by twofold, whereas lipopolysaccharide (LPS) – or staphylococcal enterotoxin B (SEB)-induced production of pro-inflammatory cytokines IL-2, IL-4, granulocyte macrophage colony stimulating factor (GMCSF), IL-6, and TNF-α was suppressed by up to fourfold (Derocq et al. 1995; Bourrie et al. 1995, 2004). This immune suppressive effect was shown to protect mice against acute and chronic inflammatory conditions such as acute graft-versus-host reaction, SEB infection, and LPS (Casellas et al. 1994; Bourrie et al. 2004). Importantly, SR31747A modulation of cytokine production was only observed in inflammatory conditions, not basal conditions. SR31747A did not appear to directly affect humoral immune responses (Bourrie et al. 1995, 1996, 2004; Casellas et al. 1994; Derocq et al. 1995).

SR31747A has cancer cell antiproliferative as well as immune suppressive properties (Bourrie et al. 2004; Casellas et al. 2004). Casellas and colleagues published a series of papers demonstrating the anti-tumor effects of SR31747A in vitro and in vivo [reviewed in (Casellas et al. 2004)]. This group reported potent

SR31747A inhibition of cancer cell proliferation in vitro, with IC_{50} values in the nanomolar range (Labit-Le Bouteiller et al. 1998). This was surprisingly potent, particularly in these 2-D in vitro assays. These results differed from most other published data demonstrating cancer cell growth and proliferation inhibition in the micromolar drug concentration range (Casellas et al. 2004).

In vivo, the anti-tumor efficacy of SR31747A was demonstrated against xenografted human breast and prostate cancer cell lines, including MCF-7, MDA-MB-231, PC3, DU145, and LNCaP. In all of these xenografted tumor studies, SR31747A was injected intraperitoneally (i.p.) at 25 mg/kg/day into immune deficient mice. SR31747A treatment resulted in similar tumor growth inhibition (TGI) of MDA-MB-231, PC3, DU145, and LNCaP xenografted tumors with TGI values of 60%, 50%, 40%, and 45%, respectively (Berthois et al. 2003). Importantly, in all of these in vivo efficacy studies, the authors observed no weight loss of mice treated with 25 mg/kg/day SR31747A for 2–3 months compared to control mice, indicating that this drug was well tolerated at efficacious doses (Berthois et al. 2003; Labit-Le Bouteiller et al. 1998).

In light of promising developments in the field of immune oncology, it would be of interest to evaluate the dual immune modulatory and cell autonomous growth inhibiting properties of compounds such as SR31747A in relevant preclinical tumor models. However, we were unable to find any published reports of this compound in syngeneic tumor models with immune competent mice.

4.2.9 BD1047

BD1047, a prototypic Sigma1 antagonist/inhibitor, is a modest inhibitor of cell proliferation in vitro. However, it appears not to be cytotoxic (Spruce et al. 2004). BD1047 is often used to selectively block the effects of agonists and thus demonstrate Sigma1-mediated pharmacology. In vivo, BD1047 has been shown to block the tumor growth promoting effects of PRE-084 in an L1C2 murine lung carcinoma tumor model (Gardner et al. 2004). BD1047 administered alone has not been shown to alter tumor growth in vivo.

In an SEB injection model, BD1047 blocked cocaine-induced IL-10 production, but had no effect on IL-10 levels in response to SEB injection when administered alone. Further, BD1047 blocked PRE-084 induction of IL-10 mRNA expression and production of IL-10 in IL-2 treated BALB/c splenocytes (Zhu et al. 2003).

4.2.10 Rimcazole (BW234U)

Rimcazole was initially evaluated in clinical trials to treat schizophrenia but did not advance primarily due to lack of efficacy (Gilmore et al. 2004; Katz et al. 2003). Rimcazole has been classified as a Sigma1 antagonist/inhibitor in part based on its inhibition of the potentiating effects of the Sigma1 agonist/activator (+)-SKF-10047 on neurogenic contractions in the mouse vas deferens and its ability to block cocaine-induced seizures and hypermotility (Matsuno et al. 1993, 1996a; Katz et al. 2003; Gilmore et al. 2004).

Spruce and colleagues proposed this compound as a potential anti-cancer agent (Spruce et al. 2004; Achison et al. 2007). Rimcazole was among a number of prototypic putative Sigma1 antagonists/inhibitors that suppressed cell proliferation and viability in cancer cell lines, with rank order potency of IPAG > rimcazole > BD1047 > reduced haloperidol > BD1063. However, several non-transformed, non-cancer cell types such as fibroblasts, primary epithelial cells, and even cerebellar granule neurons (which express high levels of Sigma1) were insensitive to the proapoptotic effects of Sigma1 antagonists/inhibitors rimcazole and IPAG (Spruce et al. 2004). In these studies, consistent with reports from most other groups, the prototypic putative Sigma1 agonists (+)-pentazocine and (+)-SKF-10047 did not inhibit cell proliferation and were not cytotoxic. Both of these Sigma1 selective putative agonists blocked the antiproliferative and proapoptotic effects of rimcazole and IPAG, demonstrating Sigma1-mediated actions of these compounds (Spruce et al. 2004).

Spruce and colleagues also showed that in vivo tumor growth was suppressed by systemic administration of rimcazole in xenografted tumor models of hormone-insensitive breast cancer (MDA-MB-231, MDA-MB-468), hormone-sensitive and hormone-insensitive prostate cancer (LNCaP, DU145), and p53-null lung carcinoma (H1299) (Spruce et al. 2004). In a separate study by Rybczynska and colleagues, daily i.p. injection of rimcazole for 2 weeks in nude mice bearing A375M human melanoma xenografts suppressed tumor weight by fourfold compared to vehicle controls, with no observable toxic side effects (Rybczynska et al. 2013).

In a subsequent publication Spruce and colleagues showed that induction of hypoxia inducible factor-1alpha (HIF-1α) contributes to rimcazole-mediated cancer cell death, at least in some instances. They demonstrated that treatment of colorectal (HCT-116) and breast (MDA-MB-231) cancer cells with rimcazole resulted in increased HIF-1α protein levels under normoxic conditions and that this is a mediator of apoptosis in this context. Furthermore, HCT-116p53+/+ cells were more sensitive than HCT-116p53−/− cells to the proapoptotic effects of rimcazole, suggesting that p53 contributes to this mechanism of action. Co-administration of (+)-pentazocine significantly attenuated rimcazole induced HIF-1α, suggesting that these effects were Sigma1-mediated (Achison et al. 2007).

In this study, RNAi knockdown of HIF-1α attenuated rimcazole induced apoptosis to comparable extents in p53 deficient and wild type cell lines; thus, in this model HIF-1α was required for rimcazole induced apoptosis (Achison et al. 2007). Of note, (+)-pentazocine also attenuated induction of HIF-1α by the anoxia mimetic deferoxamine mesylate (DFX), suggesting that promoting Sigma1 acts to suppress proapoptotic HIF-1α activity. Rimcazole did not induce HIF-1α in non-transformed, non-cancer fibroblasts or mammary epithelial cells (Achison et al. 2007).

Consistent with the proapoptotic activities of rimcazole, de Bruyn et al. reported that co-treatment with rimcazole potentiates the proapoptotic activities of the bi-functional therapeutic fusion protein, designated anti-MCSP:TRAIL [anti-melanoma chondroitin sulfate proteoglycan (MCSP):Tumor Necrosis Factor Related Apoptosis Inducing Ligand (TRAIL)]. Anti-MCSP:TRAIL was designed to bind

and accumulate at the cell surface of MCSP-positive melanoma cells, subsequently block MCSP-mediated growth signaling, and trigger apoptotic TRAIL-signaling (de Bruyn et al. 2010).

Although these in vitro and in vivo xenograft studies support the notion that pharmacological inhibition of Sigma1 is a valid approach to suppressing tumor growth, some of the potential off-target effects of rimcazole may render this particular compound difficult to develop as an anti-cancer agent. A concern with using doses of rimcazole that may be required for anti-tumor activity is that rimcazole is also a potent dopamine transporter (DAT) inhibitor. Rimcazole binds Sigma1 with low affinity and binds DAT with high affinity [reviewed in (Gilmore et al. 2004; Husbands et al. 1997; Katz et al. 2003)].

4.2.11 IPAG

(1-(4-Iodophenyl)-3-(2-adamantyl)guanidine), a prototypic Sigma1 antagonist/inhibitor, was synthesized as part of a series of N,N′-di-o-tolylguanidine (DTG) analogue radiotracers for positron emission tomography (Scherz et al. 1990; Wilson et al. 1991; Kimes et al. 1992). [^{125}I]-IPAG has been used to label and quantify Sigma1 binding sites in vivo, in situ in tissue samples, and in membrane preparations from cancer cell lines (Kimes et al. 1992; Whittemore et al. 1997; Schrock et al. 2013). Recently, a rapid method to radioiodinate [^{125}I]-IPAG was published that should facilitate future studies with this radioligand (Pickett et al. 2015).

The specificity of IPAG binding to Sigma1 has been demonstrated by multiple groups (Kimes et al. 1992; Whittemore et al. 1997; Spruce et al. 2004; Schrock et al. 2013). For example, RNAi knockdown of Sigma1 produces a corresponding decrease in [^{125}I]-IPAG radioligand binding (Schrock et al. 2013). And, blockade of IPAG by (+)-pentazocine and (+)-SKF10047 has been observed in functional assays with cancer cell lines (Spruce et al. 2004).

Spruce and colleagues reported that treatment of MDA-MB-468 and MCF-7 breast adenocarcinoma cell lines with IPAG produced a dose-dependent suppression of cell proliferation and induction of caspase-dependent apoptosis. IPAG treatment was reported to induce calcium-dependent activation of phospholipase C and calcium-independent inhibition of phosphatidylinositol 3-kinase (PI3K) pathway signaling. This effect was only observed in Sigma1 antagonist/inhibitor sensitive cells. Non-cancer cells, including cerebellar granule neurons (which express high levels of Sigma1) did not respond in this way to IPAG treatment, and normal mammary epithelial cells were insensitive to IPAG induced cell death (Spruce et al. 2004). The authors confirmed that these responses to IPAG were Sigma1-mediated by blocking with co-administration of (+)-SKF10047 and (+)-pentazocine (Spruce et al. 2004).

A series of more recent publications suggest that IPAG may function as a regulator of cancer cell protein homeostasis (Kim et al. 2012; Schrock et al. 2013; Thomas et al. 2017). Schrock et al. tested a panel of diverse ligands with affinity for Sigma1 and discovered that a subset of them induced the unfolded protein response (UPR) and autophagy in a number of cancer cell lines. Of these ligands, IPAG

emerged as a potent, Sigma1-selective inducer of UPR and autophagy. It does so in a dose- and time-responsive manner in a number of cancer cell lines including breast, prostate, pancreas, and liver carcinoma (Schrock et al. 2013).

Interestingly, treatment with Sigma1 antagonists/inhibitors did not activate irreversible signaling cascades toward cell death. On the contrary, Schrock et al. demonstrated that continuous, protracted antagonist/inhibitor treatment was required to produce cell death, and that the effects of IPAG were reversible. When IPAG was washed out of cell culture media, there was a sequential subsiding of autophagy followed by a return of UPR markers to basal levels. The mechanism underlying this process is unclear. However, if the basis of Sigma1 function is protein–protein interactions (PPIs), then the sequential reversal of Sigma1 antagonist/inhibitor actions upon removal of compound suggests that these effects require high Sigma1 occupancy and continuous ligand engagement to maintain the disruption of Sigma1 PPIs.

IPAG has been used in recent studies to show that Sigma1 ligands can selectively regulate the stability, trafficking, and signaling of oncogenic driver proteins in cancer cells. Thomas et al. demonstrated that these Sigma1-mediated actions could be exploited to suppress aberrant androgen receptor (AR) activity and protein levels in prostate cancer cells (Thomas et al. 2017). The dual goals of the Thomas et al. study were to better understand the role of Sigma1 with regard to the stabilization and function of an oncogenic protein, in this case AR, and to determine whether modulation of its activity may have therapeutic value (Thomas et al. 2017). The authors showed that IPAG blocked 5α-dihydrotestosterone (5α-DHT) induced nuclear translocation of AR and suppressed AR transcriptional activity. Treatment with IPAG also induced proteasomal degradation of AR, suppressing the protein levels of both full-length (AR) and constitutively active splice variant AR (ARV). Consistent with these data and with putative antagonist/inhibitor activity of IPAG, RNAi knockdown of Sigma1 also suppressed AR protein levels and transcriptional activity. Furthermore, in support of the importance of Sigma1 in prostate cancer cell growth and survival, RNAi knockdown of Sigma1 and treatment with IPAG both inhibited clonogenic growth and survival of prostate cancer cell lines (Thomas et al. 2017).

The study by Thomas et al. revealed a direct interaction between Sigma1 and the AR axis in prostate cancer and the in vivo efficacy of Sigma1 antagonists/inhibitors in suppressing prostate tumor growth through this mechanism (Thomas et al. 2017). The authors further demonstrated with co-immunoprecipitation experiments that Sigma1 physically associates with constitutively active ARVs (in this case, ARV7 and AR^{v567es}) as well as the hormone responsive full-length AR. Antagonists/inhibitors were able to suppress the transcriptional activity and protein levels of these constitutively active ARVs in metastatic castration resistant prostate cancer (mCRPC) cell lines, both in vitro and in vivo. In vivo, inhibition of Sigma1 with a drug-like analog of IPAG significantly inhibited the growth of xenografted 22Rv1 (ARV driven mCRPC cell line) tumors. Importantly, inhibition of tumor growth was associated with elimination of AR and ARV in responsive tumors, consistent with a Sigma1-AR/ARV mechanism-related response. Moreover, this Sigma1

antagonist/inhibitor produced no detectable side effects at efficacious doses; no weight loss and no behavioral abnormalities were observed under these study conditions (Thomas et al. 2017).

Interestingly, the authors observed no measurable change in glucocorticoid (GR) protein levels in response to IPAG treatment. Considering that AR and GR are closely related proteins with conserved sequences and mechanisms on action, this result was unexpected; however, it highlighted the selectivity of Sigma1 modulator actions. The properties of Sigma1 and specific mechanisms that underlie this selectivity remain to be determined.

Sigma1 also interacts with ErbB receptors, and in the study by Thomas et al., IPAG dose-responsively suppressed ErbB2/HER2 and ErbB3/HER3 protein levels in prostate cancer cells (Thomas et al. 2017). This is particularly relevant to prostate cancer disease progression and therapy as ErbB2 and 3 levels and activity have been reported to be upregulated in CRPC as an adaptive resistance mechanism engaged by malignant prostate cancer cells in response to treatment with standard of care AR-axis targeted therapies (Gao et al. 2016; Berger et al. 2006; Chen et al. 2010, 2011).

These data suggest that Sigma1 may play a role in feedback mechanisms that regulate AR-associated signaling networks and provide evidence in support of targeting Sigma1 to treat AR-driven cancers. Of particular interest, targeting Sigma1 in order to allosterically modulate AR is an intriguing approach that may bypass or prevent the adaptive resistance inherent to current AR-targeted therapies.

4.2.12 Donepezil

Although better known as an acetylcholinesterase inhibitor approved for the treatment of Alzheimer's disease, donepezil also binds Sigma1 with high affinity (Kato et al. 1999), and some of the cognitive benefits of donepezil have been associated with its affinity for Sigma1 (Maurice et al. 2006; Maurice 2016). In light of these observations, there is emerging interest in the potential use of donepezil to mitigate and treat cognitive impairment associated with radiotherapy and chemotherapy and improve the quality of life in patients being treated for cancer (Loh et al. 2016), particularly those with brain tumors (Correa et al. 2016; Shaw et al. 2006; Rapp et al. 2015). Recently, the results of a randomized, placebo-controlled pilot study to assess the ability of donepezil to improve specific measures of cognitive function in breast cancer patients was published. In this clinical trial, patients in the donepezil treatment group performed significantly better than the placebo administered control group on parameters of the Hopkins Verbal Learning Test-Revised (HVLT-R) regarding total recall and recognition discrimination (Lawrence et al. 2016). The benefit of donepezil-mediated attenuation of chemotherapy induced cognitive impairment was also observed in preclinical mouse models; this may provide experimental models to investigate the mechanisms underlying these beneficial effects (Winocur et al. 2011).

Additionally, preclinical studies have suggested that donepezil may also have anti-tumor properties. Donepezil was reported to promote caspase-dependent apoptosis in U937 human histiocytic lymphoma and HL-60 human promyelocytic leukemia cells (Ki et al. 2010). It has been reported to have antiproliferative and anti-migratory effects on glioblastoma cells in vitro (Megalizzi et al. 2009). Furthermore, treatment with a combination of donepezil and temozolomide prolonged survival of mice orthotopically grafted with Hs683 glioblastoma cells compared to temozolomide or donepezil alone (which did not prolong survival) (Megalizzi et al. 2009).

4.2.13 Endogenous Molecules That Bind Sigma1

Several endogenous molecules have been shown to bind Sigma1. These molecules include the steroid hormones didehydroepiandrosterone (DHEA), progesterone, and pregnenolone, as well as sphingolipid-derived amines (D-erythro-sphingosine) and cholesterols. Even *N*,*N*-dimethyltryptamine (DMT) has been proposed as a Sigma1 ligand [reviewed in (Maurice and Su 2009; Fontanilla et al. 2009; Narayanan et al. 2011)].

4.3 Relationship Between Sigma1/*SIGMAR1* Levels and Drug Response

Based on the current literature, it appears that *SIGMAR1* transcript and Sigma1 protein levels alone do not necessarily predict or correlate with cancer cell response to Sigma1 inhibitors.

Evaluation of rimcazole in the National Cancer Institute's NCI-60 screening panel revealed that rimcazole had growth inhibitory effects, with GI_{50} values for the 59 cell lines currently in this panel ranging from 1.9 to 38 μM (Spruce et al. 2004). Spruce and colleagues subsequently used transcript data from the NCI-60 associated Cell Miner gene expression database to show that sensitivity to rimcazole's antiproliferative and proapoptotic properties did not correlate with *SIGMAR1* transcript levels (Spruce et al. 2004). These data suggest that the mere presence of *SIGMAR1* or increased levels of *SIGMAR1* do not necessarily correlate with response to Sigma1 ligands (Spruce et al. 2004). In support of this notion, [^{3}H](+)-pentazocine radioligand binding studies confirmed that Sigma1 is present at relatively low levels on MCF-7 cells, and it is as sensitive to rimcazole treatment as MDA-MB-468 cells, which express a higher density of Sigma1 sites ($K_d = 7.7$ nM; $B_{max} = 3{,}250$ fmol/mg of protein) (Spruce et al. 2004).

In general, gene expression data and radioligand binding assay data show that normal, healthy tissues appear to express less *SIGMAR1* and Sigma1 binding sites than corresponding cancer tissue. However, some tissue/cell types intrinsically express high levels of *SIGMAR1* and Sigma1. For example, cerebellar granule neurons (CGN) (Starr and Werling 1994) and hepatocytes (Mei and Pasternak 2001) express high densities of Sigma1, greater than some cancer cell lines. However, Spruce and colleagues showed that although CGN express high levels

of Sigma1, they were not sensitive to the antiproliferative or cytotoxic effects of antagonists/inhibitors (Spruce et al. 2004). Mouse whole brains have Sigma1 density comparable to cancer cell lines with [^{3}H](+)-pentazocine radioligand binding B_{max} values in excess of 1,000 fmol/mg protein (Langa et al. 2003). Yet, neurotoxicity and hepatotoxicity have not been widely reported in animal studies with Sigma1 antagonists/inhibitors (see Sect. 4.5, below).

These observations, along with the general absence of cytotoxicity in preclinical animal studies of Sigma1 ligand efficacy and the Phase I safety assessment of selective Sigma1 antagonists/inhibitors (Abadias et al. 2013; Gris et al. 2016), altogether suggest a context-dependent response to Sigma1 ligands. In other words, it is possible that Sigma1 is being used differently in different organs/tissues as well as in normal physiological versus pathophysiological conditions.

The specific biochemical and molecular mechanisms underlying these potential context-dependent effects remain poorly understood. However, we propose that the preponderance of published data suggests that these mechanisms involve distinct, context-dependent Sigma1 protein associations. Thus, one explanation is that small molecule modulators of Sigma1 target Sigma1 protein complexes and not Sigma1 per se. The composition of distinct Sigma1 associated protein complexes may determine biochemical and cellular response to Sigma1 targeted drugs. This concept is illustrated in Figs. 2 and 3. This could explain, in part, the differential toxicity of Sigma1 inhibition in cancer versus normal cells. In this case, although Sigma1 is widely expressed, its stabilizing function is more heavily or differentially engaged in conditions such as the proteotoxic stress characteristic of metabolically stressed cancer cells. In contrast, normal cells appear to be markedly less sensitive to disruption by *SIGMAR1* knockout or Sigma1 antagonists/inhibitors and may be able to compensate or adapt to treatment.

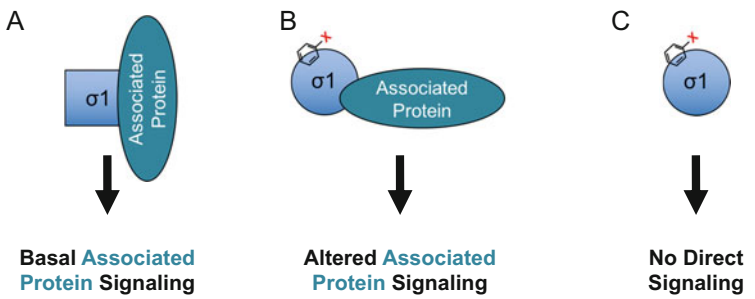

Fig. 2 Proposed model for Sigma1 ligands as allosteric modulators of protein–protein interactions. In this proposed model Sigma1 protein association, and not Sigma1 itself, determines cellular response to Sigma1 ligands. (**a**) Under basal conditions, Sigma1 binds to its associated protein(s), thus allowing for normal associated protein signaling. (**b**) Sigma1 ligand (⟨⬡⟩–x) binding to Sigma1 allosterically modulates the signaling of Sigma1 associated proteins. (**c**) Sigma1 has no known intrinsic signaling or enzymatic activity, and in the absence of associated proteins, ligand binding does not elicit direct signaling

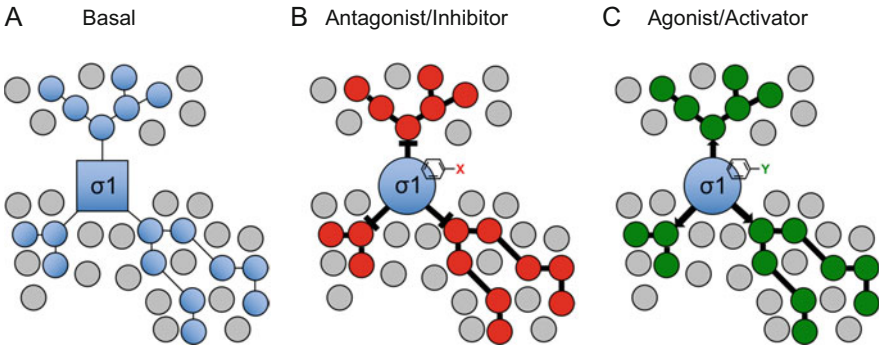

Fig. 3 Proposed model for Sigma1 as a selectively multi-functional drug target. (**a**) Under basal, steady-state conditions, Sigma1 associates with its partner proteins (●) and is surrounded by other related proteins with which it does not physically associate or regulate (○). (**b**) When a Sigma1 inhibitor/antagonist (⬡–X) binds to Sigma1, it selectively suppresses Sigma1 associated proteins and their downstream interactions and signaling pathways. (**c**) When a Sigma1 activator/agonist (⬡–Y) binds Sigma1, it promotes these associated protein pathways. Thick lines in (**b**) and (**c**) indicate increased strength of interaction. The circles directly connected to Sigma1 represent associated proteins that are physically bound to Sigma1, and indirectly connected circles represent their downstream signaling pathway components. An example of this concept is Sigma1 regulation of AR (Thomas et al. 2017)

4.4 Relationship Between Reported Ligand Binding Affinity and Functional Potency in Cell Based Assays

An important unresolved question regarding Sigma1 pharmacology in the context of cancer is how to explain apparent discrepancy between ligand binding affinity in biochemical membrane preparations and functional potency (activity) in live-cell-based functional assays. In traditional in vitro binding assays, many Sigma1 ligands bind with low nanomolar (nM) K_i/K_d whereas in cell-based functional assays, the response to Sigma1 ligands is observed at high nM to low μM concentrations. In this section, we consider a number of potential explanations.

4.4.1 High and Low Affinity Sigma1 Binding Sites

Dehydroepiandrosterone (DHEA) and DHEA sulfate (DHEAS), along with other neurosteroids including pregnenolone and progesterone, have been proposed as endogenous modulators of Sigma1; however, their relatively low binding affinity has been the source of dispute regarding this classification. The argument assumes that only the higher affinity, low nanomolar binding sites are meaningful Sigma1 pharmacological sites. However, this has not been confirmed. Some of these "low affinity" sites may be relevant and may elucidate some of the context-dependent physiological roles of Sigma1. These distinct binding sites may reflect distinct Sigma1 conformations, multi-protein complexes, or populations. Although the physiological and pharmacological relevance of these sites remains to be

determined, there is evidence, published over several decades, of higher and lower affinity Sigma1 binding sites.

Thomas et al. performed radioligand binding saturation assays on tumors and non-cancerous tissue from patients (Thomas et al. 1990). The authors detected sigma binding sites in all nine tumors tested with [^{3}H]DTG K_d values ranging from 27 to 83 nM. Interestingly, the authors report that a two-site model fit their binding data better than a one-site model, with a high affinity binding site (K_{d1}) 18–38 nM and lower affinity binding site (K_{d2}) of 165–2,880 nM (Thomas et al. 1990).

Bowen and colleagues quantified Sigma1 binding sites with [^{3}H](+)-pentazocine in crude membrane preparations from 13 cancer cell lines including C6 glioma, N1E-115 neuroblastoma, NG108-15 neuroblastoma x glioma hybrid, human T47D breast ductal carcinoma, human NCI-H727 lung carcinoid, and human A375 melanoma (Vilner et al. 1995b). The authors identified two distinct Sigma1 binding sites in most of these cancer cell lines, high affinity (K_{d1} = 0.67–7.0 nM) with B_{max1} = 25–108 fmol/mg protein, and low affinity sites (K_{d2} = 127–600 nM) with B_{max2} = 942–5,431 fmol/mg protein. Interestingly, the low affinity site was more abundant than the high affinity site in the cancer cell lines in this study (Vilner et al. 1995b).

Wu et al. described a low affinity Sigma1 binding site in intact NCB-20 (mouse neuroblastoma x Chinese hamster brain hybrid) cells (Wu et al. 1991). This group found that [^{3}H](+)-SKF10047 binds two populations of binding sites in intact NCB-20 cells, a higher affinity binding site (K_d = 49 nM, B_{max} = 1.0 pmol/mg protein) and a lower affinity binding site (K_d = 9.6 μM, B_{max} = 69 pmol/mg protein). The rank order potencies of a number of sigma ligands at the lower affinity site correlated (using Spearman rank correlation) with the electrophysiological assay potencies both in this study and in a previously reported study using a guinea pig vas deferens assay (Vaupel and Su 1987). These data indicated that the electrophysiological responses at high and low affinity binding sites were the result of Sigma1 occupancy. The authors of this study noted that it was unclear whether the high and low affinity Sigma1 binding sites represented two separate receptors or the same receptor with two different states (Wu et al. 1991).

More recently, Safrany and colleagues described high and low affinity Sigma1 binding sites or conformations in the Sigma1-positive MDA-MB-468 breast adenocarcinoma cell line (Brimson et al. 2011). When a model assuming single-site binding was used, only the high affinity, 2.5 nM binding site was detected. However, when a multiple-site model was used, IPAG displaced [^{3}H](+)-pentazocine with a K_i of 8 μM (Brimson et al. 2011), which corresponds to concentrations at which activity is detected in cell-based assays of cancer (Spruce et al. 2004; Kim et al. 2012; Schrock et al. 2013; Thomas et al. 2017).

Spruce and colleagues noted that rimcazole displaces [^{3}H](+)-pentazocine with an IC_{50} of 2.7 ± 1.8 μM, which is close to its IC_{50} in MDA-MB-468 cell proliferation and survival assays (Spruce et al. 2004). Interestingly, this suggests that

rimcazole only binds the putative low affinity Sigma1 binding site or conformation (Spruce et al. 2004). It is noteworthy that the reported binding affinity of rimcazole to Sigma1 ranges from the high nanomolar to low micromolar range (see Table 2).

Similarly, Wilke et al. reported that iodoazidococaine (IAC), a Sigma1 photoprobe, inhibited voltage-activated potassium current (IK) in DMS-114 (small cell lung carcinoma) cells. IAC photolabeling of Sigma1 in cell homogenates was inhibited by (+)-SKF10047 with an IC_{50} of 7 μM. This was similar to the half-maximal concentration of (+)-SKF10047 that inhibited IK (14 μM) (Wilke et al. 1999).

4.4.2 Cell Penetration

One possible explanation is that the cell plasma membrane limits access to intracellular Sigma1 binding sites. Published K_d and K_i values of Sigma1 ligands are based on binding assays performed with membrane preparations or in some instances with permeabilized cells. Does facilitating compound entry increase functional potency? The availability of sufficient free compound within the cell to act on intracellular targets such as Sigma1 may also explain why the effective concentrations of many Sigma1 ligands are significantly higher in cell-based functional assays than their binding affinities – which are largely determined with biochemical membrane preparations and not intact cells.

Although the answer to this question remains unanswered, at least one report suggests that cell penetration may be a contributing factor to functional potency. Banerjee and colleagues (Pal et al. 2011) have reported that facilitating cell entry by conjugating haloperidol with cationic lipids of varying chain lengths increases the functional potency of haloperidol in in vitro cell proliferation and cytotoxicity assays. For example, HP-C8, a cationic lipid-modified haloperidol analogue with a lipid chain of 8 carbon atoms was >100-fold more potent than haloperidol in inhibiting the proliferation and survival of MCF-7 and MDA-MB-231 breast cancer cells. HP-C8 was a two- to threefold more potent inducer of apoptosis in these cancer cells compared to non-transformed COS-1 and HEK293 cells. The authors reported that HP-C8 was also efficacious in vivo. Xenografted mice bearing B16F10 melanoma tumors produced a threefold reduction in tumor growth following 5 intraperitoneal injections of 7.5 mg/kg HP-C8 at 2- to 3-day intervals (Pal et al. 2011).

4.5 Safety of Treatment with Sigma1 Ligands

Because Sigma1 is broadly expressed in tissues throughout the body, the safety of Sigma1 modulators is a common concern. However, there is little empirical or clinical evidence to support target-mediated toxicity associated with Sigma1 selective compounds. Indeed, it has been well documented in the literature that

compounds that are active at Sigma1 are generally safe (Abadias et al. 2013; Gris et al. 2016; Nieto et al. 2012; Zamanillo et al. 2013; Luedtke et al. 2012; Blasio et al. 2015; Cendan et al. 2005a; Romero et al. 2012; Maurice and Su 2009; Spruce et al. 2004; Casellas et al. 2004; Riganas et al. 2012a, b, c; Moody et al. 2000; Thomas et al. 2017).

One salient piece of evidence that Sigma1 inhibition is generally benign is that *SIGMAR1* knockout (KO) mice are viable, fertile, and do not display a phenotype overtly different from wild type mice (Langa et al. 2003), which supports the notion that inhibiting Sigma1 has minimal impact on normal tissues. This raises a separate question regarding potential compensatory mechanisms that may be engaged when *SIGMAR1* is eliminated; however, such mechanisms have not yet been identified.

Pharmacological inhibition of Sigma1 appears to be safe (benign) as well. Most recently, clinical trials of the Sigma1 antagonist/inhibitor S1RA have demonstrated lack of toxicity in humans (Abadias et al. 2013; Gris et al. 2016). S1RA (also known as E-52862) was evaluated in single- and multiple-dose phase I clinical studies and demonstrated positive safety, tolerability, and pharmacokinetic profiles in healthy human subjects (Abadias et al. 2013). Of the 175 subjects enrolled, no serious adverse events were observed, and no clinically significant changes were observed in electrocardiogram (ECG), Holter monitoring, vital signs, and laboratory assessments. This Sigma1 antagonist/inhibitor is currently in phase II clinical trials for treatment of neuropathic pain of different etiology using a daily oral dose of 400 mg (Abadias et al. 2013; Gris et al. 2016).

Consistent with this observation, in a number of published tumor xenograft studies, no adverse events (including weight loss and behavioral abnormalities) were observed at efficacious doses of Sigma1 antagonists/inhibitors (Spruce et al. 2004; Casellas et al. 2004; Riganas et al. 2012a, b, c; Moody et al. 2000; Thomas et al. 2017).

Based on their antiproliferative and cytotoxic effects on cancer cells and tumors, another common concern is whether Sigma1 antagonists/inhibitors have the potential to promote neurodegeneration (Tsai et al. 2014). As with the general safety concerns, there is little empirical or clinical evidence demonstrating that Sigma1 selective antagonists/inhibitors promote neurodegeneration or exacerbate symptoms of neurodegenerative disease. At the cellular level, cerebellar granule neurons, which express higher levels of Sigma1 than many cancer cells, were not sensitive to the antiproliferative or cytotoxic effects of Sigma1 antagonists in at least one report (Spruce et al. 2004). In behavioral models focusing on cognitive deficits, Sigma1 antagonists/inhibitors did *not* worsen symptoms, and did *not* promote symptoms (Matsumoto et al. 1995; Maurice et al. 1994, 1998). In most published studies, antagonists were used to block the effects of agonists and demonstrate their Sigma1-mediated actions. However, when administered alone, the antagonists generally manifested no effect in rodent models of behaviors associated with Alzheimer's disease. This has been demonstrated in a number of studies (Wang et al. 2003; Espallergues et al. 2007; Villard et al. 2009; Yang et al. 2012; Maurice 2016).

5 Sigma1: Receptor, Chaperone, or Scaffold?

It is becoming increasingly clear that Sigma1 is not a traditional receptor. Although it remains unclear whether Sigma1 should be defined as a chaperone or scaffolding protein in cancer cells, the absence of clear enzymatic or signaling activity of Sigma1 along with its association with and modulation of diverse signaling molecules are evidence in support of Sigma1 as a scaffolding protein. Scaffolds have no enzymatic or signaling activity; however, they physically interact with other proteins to assemble, localize, and regulate signaling complexes. They coordinate the organization of signaling or chaperone molecules into discrete complexes to facilitate efficient and specific activity (Good et al. 2011; Bauer and Pelkmans 2006). Scaffolding proteins can allosterically modulate signaling or enzymatic activity as well as coordinate the activity of chaperones such as HSP70 and HSP90 (Cesa et al. 2015; Good et al. 2011). Scaffolds can also be inhibitory by blocking protein–protein and protein–lipid interactions (Good et al. 2011; Bauer and Pelkmans 2006). They are flexible platforms that can form multiple oligomeric conformations that comprise combinatorial assemblies of protein interaction domains that enable regulation of diverse biological processes. Consistent with recently published reports, our data suggest that Sigma1 is present as oligomers (Gromek et al. 2014; Schmidt et al. 2016). These oligomeric structures may also be a determinant of how Sigma1 forms multi-protein complexes. As a potential membrane bound scaffolding protein, Sigma1 is reminiscent of caveolins and tetraspanins (Bauer and Pelkmans 2006; Patel et al. 2008; Hemler 2014).

We propose that Sigma1 is a ligand-regulated scaffolding protein that engages in selective protein interactions. We have found that Sigma1 physically and functionally interacts with AR and ErbB-2 and -3 receptors and that these receptors are regulated by Sigma1 ligands (Thomas et al. 2017). Our data, along with published reports from other groups, suggest that Sigma1 engages in a number of multi-protein complexes, and the composition of these protein complexes appears to be context-dependent. It remains to be determined whether Sigma1 modulators directly alter PPIs or the intracellular transport and localization of Sigma1-associated protein complexes. The biochemical mechanisms and protein determinants that dictate Sigma1 PPIs have not yet been clearly defined. Therefore, the mechanistic basis of Sigma1 partner and client protein selectivity is unknown. This is a crucial missing link to understanding the complex pharmacology of Sigma1.

6 Sigma1 as a Multifunctional Drug Target

Whether Sigma1 is eventually classified as a scaffolding protein or chaperone, it is already clear that it engages in a range of heterogeneous but selective functional protein interactions (illustrated in Fig. 3). Sigma1 modulators alter multiple processes and systems in cancer cells by targeting distinct Sigma1 associated protein complexes that appear to assemble in a context-dependent manner. The known

biochemical properties and cellular activities of Sigma1 are consistent with a role as a component of the cancer cell support machinery [concept reviewed in (Dobbelstein and Moll 2014)]. Importantly, Sigma1 inhibitors are not pleiotropic, and they suppress or alter oncogenic proteins and pathways by a mechanism distinct from other drugs that target the cancer cell support machinery (Thomas et al. 2017). With respect to Sigma1 drug discovery and pharmacology, a key challenge is to understand how to harness the selective multifunctionality of Sigma1 as a drug target.

6.1 Cell Intrinsic Signaling and Activities

Multifunctional drug targets such as Sigma1 can have a number of advantages over single target therapies in regulating cell intrinsic signaling and processes. Specific targeted therapies such as tyrosine kinase inhibitors, selective receptor antagonists, and targeted monoclonal antibodies are prone to adaptive, acquired drug resistance (Komarova and Wodarz 2005; Bozic et al. 2012; Pao et al. 2005; Schwartz et al. 2015). In contrast, Sigma1 modulators used alone or in combination with targeted therapeutic agents may delay or even bypass such resistance.

In the case of prostate cancer, the inevitable resistance to androgen receptor (AR)–targeting agents is associated with reactivation of the AR axis through induction of intratumoral steroidogenesis, increased expression of AR, gain-of-function mutant AR, and constitutively active AR splice variants (Mostaghel et al. 2014; Knudsen and Kelly 2011; Attard et al. 2016; Ferraldeschi et al. 2015; Bambury and Scher 2015). This is further complicated by compensatory upregulation or feedback regulation of associated pathways such as ErbB receptor upregulation and PI3K (phosphatidyl inositol-3-kinase) activation in PTEN (*p*hosphatase and *ten*sin homolog) deficient prostate cancers (Gao et al. 2016; Carver et al. 2011). For prostate cancer, these examples demonstrate the importance of discovering and developing novel approaches to co-targeting the AR axis and the networks on which it depends.

Recently, Thomas et al. showed that three CRPC lines (C4-2, VCaP, and 22Rv1) evaluated were all responsive to small molecule Sigma1 inhibition. AR levels were suppressed in C4-2 cells and AR and ARV levels were suppressed in the AR splice variant driven VCaP and 22Rv1 cell lines. In vitro colony formation of all three lines was dose-responsively suppressed by treatment with IPAG (Thomas et al. 2017). IPAG also reduced ErbB2/HER2 and ErbB3/HER3 protein levels (Thomas et al. 2017), thus abrogating the compensatory upregulation of ErbB2/HER2 and ErbB3/HER3 that occurs in response to AR-targeted therapies (Carver et al. 2011; Mostaghel et al. 2014; Gao et al. 2016).

PTEN deficiency, by mutation or loss of PTEN, has a significant impact on prostate cancer progression. Indeed, over 50% of advanced prostate cancers are PTEN deficient (Li et al. 1997; Mulholland et al. 2011; Carver et al. 2011). Small

molecule Sigma1 inhibitors suppress growth pathway signaling in PTEN mutant LNCaP and C4-2 and PTEN null PC3 cells (Kim et al. 2012; Thomas et al. 2017). These data suggest that Sigma1 inhibitors can engage mechanisms downstream of PTEN or mechanisms that cooperate with but are distinct from canonical PI3K/Akt growth and survival signaling pathways. The ability to suppress growth signaling in PTEN deficient cancers (Kim et al. 2012; Schrock et al. 2013; Thomas et al. 2017) as well as the ability to suppress compensatory mechanisms that emerge in response to AR-targeted inhibition demonstrates that Sigma1 ligands may provide a way to bypass or suppress the redundancies and complex feedback mechanisms that render current therapeutic approaches to target growth regulatory pathways susceptible to resistance (She et al. 2010; Carver et al. 2011; Zhang and Yu 2010; Hsieh et al. 2011; Mostaghel et al. 2014; Gao et al. 2016).

Thus, the ability to pharmacologically modulate multifunctional targets such as Sigma1 is advantageous in cancer, as it imposes a barrier to compensatory response mechanisms to targeted therapies without the broad and often toxic effects of chemotherapy.

6.2 Immunomodulation

The multifunctionality of Sigma1 as a drug target may extend beyond cell intrinsic signaling and regulation of oncogenic driver proteins and pathways. For example, a series of papers in the late 1990s and early 2000s have reported immunomodulatory effects of Sigma1 ligands (Bourrie et al. 1995, 1996, 2002, 2004; Carayon et al. 1995, 1996; Derocq et al. 1995; Gardner et al. 2004; Zhu et al. 2003). These papers, which describe the cytokine modulating effects of SR31747A, PRE-084, and (+)-SKF10047, are discussed in Sect. 4, above. In summary, this work demonstrates that Sigma1 agonists/activators promote tumor growth, in part by suppressing anti-tumor immunity. However, these studies stopped short of evaluating the ability of Sigma1 antagonists/inhibitors to promote anti-tumor immunity. Although prototypic Sigma1 antagonists/inhibitors were used to block the immune suppressive and tumor promoting effects of Sigma1 agonists/activators, the direct effects of Sigma1 antagonists/inhibitors on anti-tumor immunity were not determined.

Recently, we discovered that the Sigma1 agonist/activator (SA4503) and antagonist/inhibitor (IPAG) differentially regulate the stability, trafficking, and activity of the checkpoint molecule programmed death-ligand 1 (PD-L1, also known as B7-H1 and CD274). We found that IPAG induced autophagic degradation of PD-L1 in androgen independent prostate cancer (PC3) and triple negative breast cancer (MDA-MB-231) cell lines. This resulted in decreased functional PD-L1 at the surface of these cancer cells. Consistent with this effect, RNAi knockdown of Sigma1 resulted in decreased PD-L1 levels. Conversely, treatment with SA4503 blocked these IPAG-mediated effects, and SA4503 alone promoted increased cell surface PD-L1 levels (Maher et al., unpublished data).

Taken together, these data suggest that pharmacological modulation of Sigma1 can regulate PD-L1 production and activity via immune response-induced

cytokine-mediated extracellular feedback loops as well as directly, via cell intrinsic mechanisms. Thus, Sigma1 ligands may be used as regulators of the tumor microenvironment.

6.3 Cancer-Associated Pain

Sigma1 has been extensively investigated in pain. For recent, detailed reviews of the subject see the chapters in this volume by Pasternak (*Allosteric Modulation of Opioid G-Protein Coupled Receptors by Sigma$_1$ Receptors*) and by Merlos et al. (*Sigma1 Receptor and Pain*). A number of studies over several decades have demonstrated that Sigma1 antagonists/inhibitors, but not agonists/activators, can potentiate opioid analgesia, and some Sigma1 antagonists/inhibitors produce analgesia on their own. The precise biochemical mechanism by which Sigma1 antagonists/inhibitors produce analgesia remains unclear. However, consistent with the antinociceptive effects of pharmacological inhibition, *SIGMAR1* KO mice (Langa et al. 2003) have demonstrated a decreased sensitivity to neuropathic pain in preclinical murine models (Cendan et al. 2005a, b; Entrena et al. 2009; de la Puente et al. 2009; Tejada et al. 2014). A potent and safe Sigma1 antagonist/inhibitor, S1RA (also known as E-52862), is currently in phase II clinical trials as a non-opioid analgesic, providing clinical proof of concept of safety and efficacy (Abadias et al. 2013; Gris et al. 2016; Zamanillo et al. 2013; Romero et al. 2016) (also see Sect. 4.5, above).

Sigma1 pharmacology has not been well studied in the context of cancer pain. However, a few preliminary reports suggest that Sigma1 antagonists/inhibitors may be effective analgesics to treat neuropathic pain associated with cancer (Nieto et al. 2012, 2014; Zamanillo et al. 2013). Cancer-associated pain can be mechanical, caused by pressure of a growing tumor on nerves, bone, and other tissue (Glare et al. 2014). It also can be caused by damage to nerves that can occur with treatments such as chemotherapy, radiotherapy, and surgery.

Nieto et al. compared the ability of paclitaxel to induce cold and mechanical allodynia in *SIGMAR1* KO and wild type (WT) *SIGMAR1* mice. They demonstrated that whereas cold and mechanical allodynia were similar in KO and WT mice, treatment with paclitaxel only produced these forms of allodynia in WT mice. Consistent with the absence of paclitaxel-induced neuropathy in *SIGMAR1* KO mice, administration of the Sigma1 antagonists/inhibitors BD1063 and S1RA prior to paclitaxel prevented both cold and mechanical allodynia in *SIGMAR1* WT mice. Furthermore, administration of BD1063 and S1RA after the onset of allodynia reversed paclitaxel-induced neuropathic pain (Nieto et al. 2012, 2014).

Pain associated with bone metastatic tumors is particularly problematic with myelomas and with lung, prostate, and breast cancers (Lozano-Ondoua et al. 2013; Suva et al. 2011; Roodman 2004; Mundy 2002). To evaluate the potential analgesic properties of Sigma1 antagonists/inhibitors, Zhu et al. implanted Walker 256 rat mammary carcinoma cells into the tibia of Sprague–Dawley rats to induce bone cancer pain. Administration of BD1047 attenuated mechanical allodynia.

Interestingly, Sigma1 expression in the spinal cord was elevated in tumor bearing rats compared to control (sham) rats (Zhu et al. 2015a). The Walker 256 rat mammary carcinoma cell bone pain model is reviewed elsewhere (Shenoy et al. 2016; Zhu et al. 2015b; Slosky et al. 2015).

These data raise the question, can antineoplastic small molecule Sigma1 antagonists/inhibitors also be analgesic in the context of cancer-associated pain? A compound that integrates these properties of Sigma1 pharmacology has yet to be reported.

7 Conclusions and Perspectives

A principal take-away message of this review is that the pharmacology of Sigma1 is complex, and there is still much to be done to define the mechanisms of action of Sigma1 ligands. Although their classification as agonists and antagonists is still commonly used in the literature (including this review), these putative pharmacological activities have remained undefined at the molecular level and may be inaccurate designations. Insights into the specific pharmacological and biochemical mechanisms by which Sigma1 ligands suppress cancer cell growth and survival are just beginning to emerge. As Sigma1 has no clearly defined enzymatic or signaling activity, most cellular responses to Sigma1 ligands are defined by the proteins and/or cellular systems engaged by Sigma1 (illustrated in Figs. 2 and 3). Thus, it may be more accurate to describe compounds with activity at Sigma1 as allosteric modulators of Sigma1 associated proteins (as illustrated in Fig. 2).

The concept of Sigma1 is rapidly evolving. A growing body of evidence supports the notion that Sigma1 is a novel chaperone or scaffolding protein that engages in diverse but selective protein interactions (see Sects. 4 and 5). Given the number of proteins with which it interacts, it is likely that Sigma1 has multiple "innate" functions. However, although Sigma1 modulators alter multiple processes and systems in cancer cells, the effects of Sigma1 ligands are not pleiotropic (see Sect. 4). Thus, Sigma1 is a selectively multifunctional drug target (concept illustrated in Fig. 3).

Multifunctional drug targets such as Sigma1 can have a number of advantages over single activity targeted therapies, which are prone to adaptive drug resistance (Komarova and Wodarz 2005; Bozic et al. 2012; Pao et al. 2005; Schwartz et al. 2015). In contrast to specific target-based therapies such as tyrosine kinase inhibitors, selective receptor antagonists, and monoclonal antibodies, Sigma1 modulators used alone or in combination with these agents may prolong or even prevent drug resistance. Most complex pathologies and disorders, including cancer, are not usually driven by a single cellular factor. Indeed, cancer is a heterogeneous, highly adaptive, and constantly evolving disease. Consequently, drug resistance in cancer is often accelerated by the targeted agents designed specifically to suppress individual oncogenic driver proteins. Therefore, a major challenge is to address not only the primary, existing target, but also latent targets that emerge as a result of mutations or other adaptive, compensatory mechanisms. This, of course, is the

rationale behind drug combinations. However, the potential efficacy of combining multiple targeted drugs must be balanced against potential adverse drug–drug interactions and differences in drug metabolism and pharmacokinetic (DMPK) properties that can add to the complexity of designing combinations. The development of a single drug addressing an array of targets (i.e., polypharmacology) also poses several challenges as well as advantages (Antolin et al. 2016; Azmi 2013). Modulation of Sigma1 would enable the selective inhibition of multiple nodes through one drug target (Fig. 3). Harnessing the strengths of these approaches would offer promising new possibilities to enhance therapeutic efficacy and bypass or prevent drug resistance.

Additionally, a number of studies demonstrate that Sigma1 modulators are not necessarily cytotoxic agents, and that they may be considerably more versatile (see Sects. 4 and 6). It is tempting to speculate that certain Sigma1 modulator compounds may be used not only as antineoplastic agents, but also to improve the quality of life of cancer patients, with decreased side effects and even benefits such as attenuation of cancer-associated pain (see Sect. 6).

Despite the number of studies suggesting that it is a valid drug target, there still are no Sigma1 drugs in the clinic to treat cancer. This is in large part because fundamental questions regarding the mechanism of action of Sigma1 modulators in the context of cancer remain unanswered or only partially answered. To understand how to use Sigma1 modulators for therapeutic benefit in cancer, there is a need for more detailed and definitive studies leading to a deeper understanding of Sigma1's role in tumor biology.

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Molecular Probes for Imaging the Sigma-2 Receptor: In Vitro and In Vivo Imaging Studies

Chenbo Zeng, Elizabeth S. McDonald, and Robert H. Mach

Contents

Abstract

The sigma-2 (σ_2) receptor has been validated as a biomarker of the proliferative status of solid tumors. Therefore, radiotracers having a high affinity and high selectivity for σ_2 receptors have the potential to assess the proliferative status of human tumors using noninvasive imaging techniques such as Positron Emission Tomography (PET). Since the σ_2 receptor has not been cloned, the current knowledge of this receptor has relied on receptor binding studies with the radiolabeled probes and investigation of the effects of the σ_2 receptor ligands on tumor cells. The development of the σ_2 selective fluorescent probes has proven to be useful for studying subcellular localization and biological functions of the σ_2 receptor, for revealing pharmacological properties of the σ_2 receptor ligands, and for imaging cell proliferation. Preliminary clinical imaging studies with [^{18}F]ISO-1, a σ_2 receptor probe, have shown promising results in cancer

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patients. However, the full utility of imaging the σ_2 receptor status of solid tumors in the diagnosis and prediction of cancer therapeutic response will rely on elucidation of the functional role of this protein in normal and tumor cell biology.

Keywords
Breast cancer • Cell proliferation • Confocal microscopy • Positron emission tomography • Sigma-2 receptor

1 Introduction

Sigma (σ) receptors represent a class of proteins that were initially identified as a subtype of the opiate receptors. Subsequent studies revealed that they represented a distinct class of receptors that are located in a variety of tissues and organs including the central nervous system (Hellewell et al. 1994; Walker et al. 1990). Two separate σ receptors, σ_1 and σ_2 receptors, were distinguished based on differences in the binding profile of benzomorphan compounds and respective molecular weights based on photoaffinity labeling. σ_1 receptors have a molecular weight of ~25 kDa, whereas σ_2 receptors have a molecular weight of ~21.5 kDa (Rothman et al. 1991). The σ_1 receptor has been cloned and displays a 30% sequence homology with the enzyme, yeast C8-C7 sterol isomerase (Seth et al. 1997; Hanner et al. 1996), but this receptor lacks C8-C7 isomerase activity. Studies have shown that some neuroactive steroids bind with moderate affinity to σ_1 sites and suggested that σ_1 receptors may modulate the activity of GABA and NMDA receptors in the CNS (Maurice et al. 1996, 1997; Romieu et al. 2003). More recently, the s_1 receptor was classified as a receptor chaperone which forms a complex with the inositol triphosphate receptor at the endoplasmic reticulum (ER) membrane, thereby regulating ER-mitochondrial Ca^{2+} signaling and cell survival (Tsai et al. 2009). Since the σ_2 receptor has not been cloned, most of what is known regarding the biology, function, and subcellular distribution of this receptor has been obtained through the use of in vitro receptor binding studies in tissues having a high receptor expression, and fluorescent microscopy studies in cancer cells under cell culture conditions.

2 Relationship Between the Density of σ_2 Receptors and Measures of Cell Proliferation

The first group to report that sigma receptors were overexpressed in cancer cells was Bem et al. (1991), who demonstrated that there is a twofold higher expression of σ receptors in tumor cells than in nonmalignant tissue. Vilner et al. (1995) later reported a high density of σ_2 receptors in a panel of murine and human tumor cells grown under cell culture conditions. The density of σ_2 receptors in the cancer cells was generally much higher than that of the σ_1 receptor. These results suggested that the σ_2 receptor may function as a biomarker for differentiating solid tumors from the surrounding, normal tissues. However, this study did not explore the relationship between the density of σ_2

receptors and the two key properties of cell proliferation, *proliferative status* and *proliferation rate*. The proliferative status of cancer cells is defined as the ratio of proliferating (P) cells to quiescent (Q) cells in either a solid tumor or cell culture dish (i.e., the P:Q ratio). Proliferating cells are driven into a quiescent state by either nutrient deprivation and/or hypoxia in a solid tumor or by nutrient deprivation or contact inhibition under cell culture conditions. The second property of cell proliferation, proliferation rate, refers to the time it takes a proliferating (P) cell to pass through the four different phases of the cell cycle, G1, S, G2, and M phase. Since quiescent cells are not dividing, they are typically described as being in G0 phase.

In order to determine the relationship between the density of σ_2 receptors and the proliferative status of cancer cells, Wheeler et al. used the mouse mammary adenocarcinoma cell line 66 (Wallen et al. 1984a, b) to determine if there was a difference in the density of σ_2 receptors between proliferating (66P) and quiescent (66Q) tumor cells under cell culture conditions. They observed that the density of σ_2 receptors in 66P cells was ~10 times greater than the density measured in 66Q cells (Fig. 1a) (Mach et al. 1997). The density of σ_2 receptors in 66P cells was

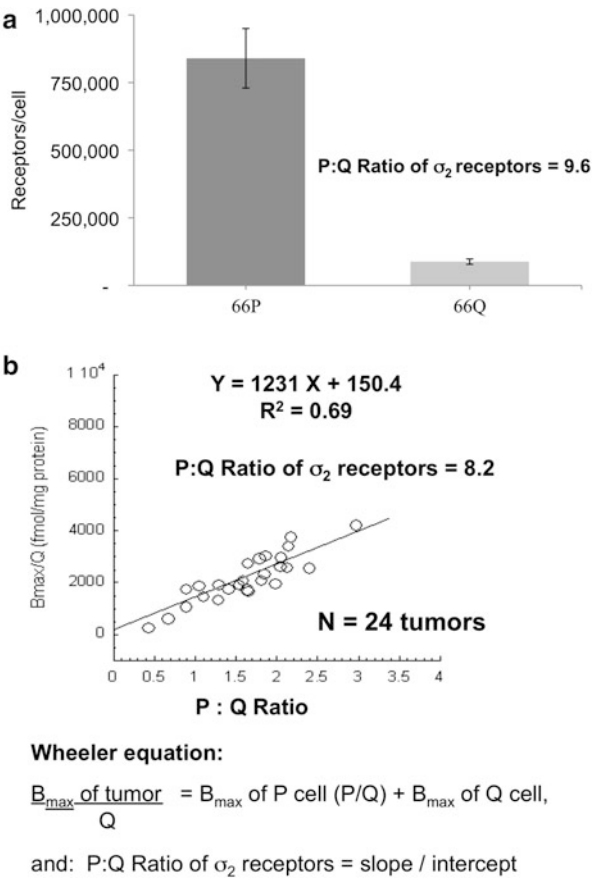

Fig. 1 The σ_2 receptor densities in proliferating and quiescent 66 tumor cells under cell culture conditions (**a**) or in solid tumor xenografts (**b**)

~900,000 copies/cell versus ~90,000 receptors/cell in the 66Q cells. This group also reported that the expression kinetics of σ_2 receptors follows the growth kinetics of the 66 cells (Al-Nabulsi et al. 1999). Since it took ~4 days for the σ_2 receptor to downregulate in the 66Q cells, the density of the σ_2 receptor appears to be independent of the phase of the cell cycle. Using a graphical method that correlates the density of σ_2 receptors with the P:Q ratio of a tumor determined by flow cytometry (i.e., the Wheeler equation), a similar P:Q ratio of σ_2 receptor density was observed in solid tumor xenografts of 66 tumors (Fig. 1b) (Wheeler et al. 2000; Shoghi et al. 2013). These results indicate that the σ_2 receptor is a biomarker of the *proliferative status* of cancer cells and the density of the receptor is independent of the *proliferation rate*. Therefore, σ_2 receptor radiotracers have the potential to measure the proliferative status of human breast tumors using noninvasive imaging techniques such as Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). Since many cancer cells of different origin have a high density of σ_2 receptors (Vilner et al. 1995), it is likely that this approach can be extended to assess the proliferative status of other human tumors, including head and neck, melanoma, and lung tumors.

3 Identification of σ_2 Receptor Selective Ligands

Although a number of structurally diverse compounds have been shown to possess a high affinity to σ receptors (Walker et al. 1990), most of these compounds bind selectively to the σ_1 receptor or have similar affinities to both σ_1 and σ_2 receptors. The first σ_2 selective ligand reported was the benzomorphan-7-one analog, CB-64D (Fig. 2) (Bowen et al. 1995a). This compound was synthesized as part of a structure–activity relationship (SAR) study aimed at improving the affinity of (−)-2-methyl-5-(3-hydroxyphenyl)morphan-7-one for μ versus κ opioid receptors (Bertha et al. 1994). A second class of compounds having a high affinity for σ_2 receptors are the 3-(ω-aminoalkyl)-1H-indole analogs (Perregaard et al. 1995; Moltzen et al. 1995). These compounds were originally designed to be serotonin 5-HT_{1A} agonists, but SAR studies resulted in the identification of Lu 28-179 (Soby et al. 2002), also known as siramesine, which has a high affinity for σ_2 receptors and a 140-fold selectivity for σ_2 versus σ_1 receptors. Other compounds that were reported to have a higher affinity for σ_2 versus σ_1 receptors are: (1) the hallucinogen, ibogaine (Bowen et al. 1995b; Mach et al. 1995), (2) the mixed serotonin 5-HT_3 antagonist/5-HT_4 agonist BIMU-1 (Bonhaus et al. 1993), (3) the tropane analog SM-21 (Mach et al. 1999; Ghelardini et al. 2000), (4) the trishomocubane analog ANSTO-19 (Nguyen et al. 1996), (5) the piperazine analog PB28 (Azzariti et al. 2006), and (6) the conformational-flexible benzamide analog YUN252 (Mach et al. 2004). Many of these structures have served as the lead compounds for fluorescent probes and radioligands for imaging σ_2 receptors both in vitro and in vivo.

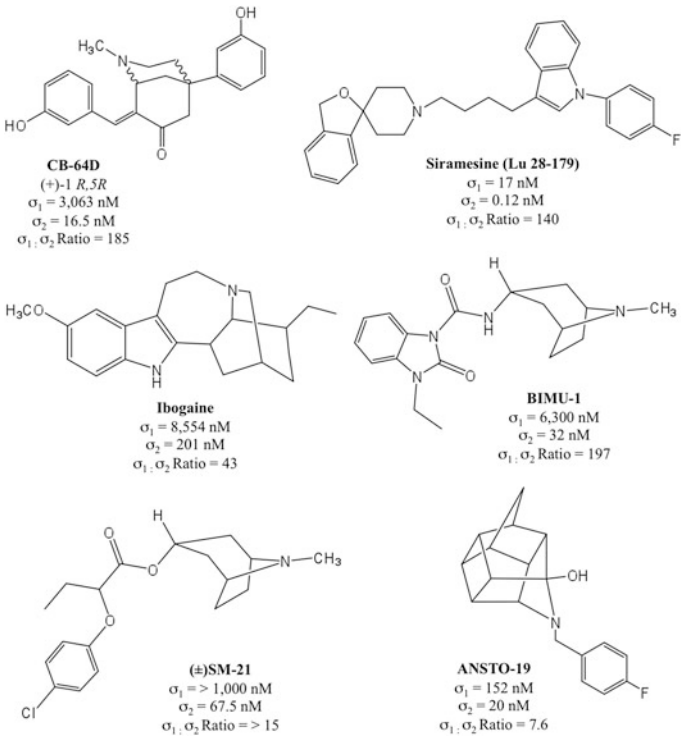

Fig. 2 Structures of σ_2 receptor ligands in the literature

4 Development of Fluorescent Probes for Studying the Subcellular Distribution of σ_2 Receptors

A number of SAR studies using BIMU-1 as the lead compound have resulted in the identification of high affinity, high selectivity σ_2 receptor ligands (Vangveravong et al. 2006; Ariazi et al. 2006; Arttamangkul et al. 2000). The most promising analog from the initial SAR study was the compound SV-119 which had a σ_2 receptor affinity of 5.2 nM and a σ_2:σ_1 selectivity of ~275 (Fig. 3a) (Ariazi et al. 2006). SV-119 was used as a lead compound for the development of the fluorescent probes, K05-138, SW120, SW107, and SW116 that have proven useful in two-photon and confocal microscopy studies of σ_2 receptors in tumor cells growing under cell culture conditions (Zeng et al. 2007, 2011). This was accomplished by the recognition that introduction of a spacer group onto the bridgehead nitrogen group in SV-119 allowed for the introduction of bulky large fluorescent moieties with only a modest loss in affinity for the σ_2 receptor.

A second compound which has proven to be useful in developing fluorescent probes for σ_2 receptors is PB28. In this case, introduction of a spacer group into the

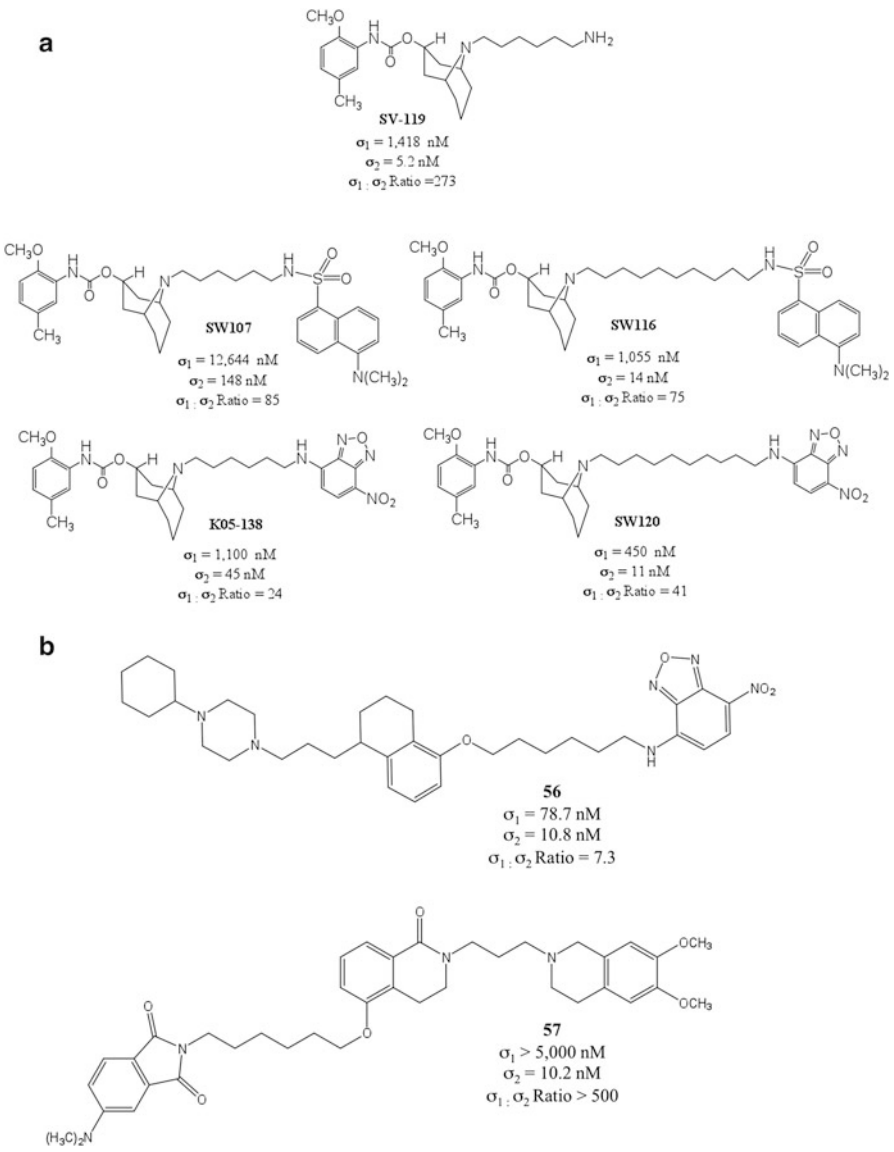

Fig. 3 Structures of SV-119 and the fluorescent probes, K05-138, SW120, SW107, and SW116 (**a**). Structures of the fluorescent probes based on PB28 and the 3,4-dihydroisoquinoline-1-one scaffold (**b**)

5-methoxy group enabled the introduction of fluorescent moieties having a high affinity for σ_2 receptors and a good σ_1:σ_2 selectivity ratio (Abate et al. 2011). Another promising fluorescent probe for studying the σ_2 receptor utilized the 3,4-dihydroisoquinoline-1-one scaffold (Niso et al. 2015) (Fig. 3b). Examples of

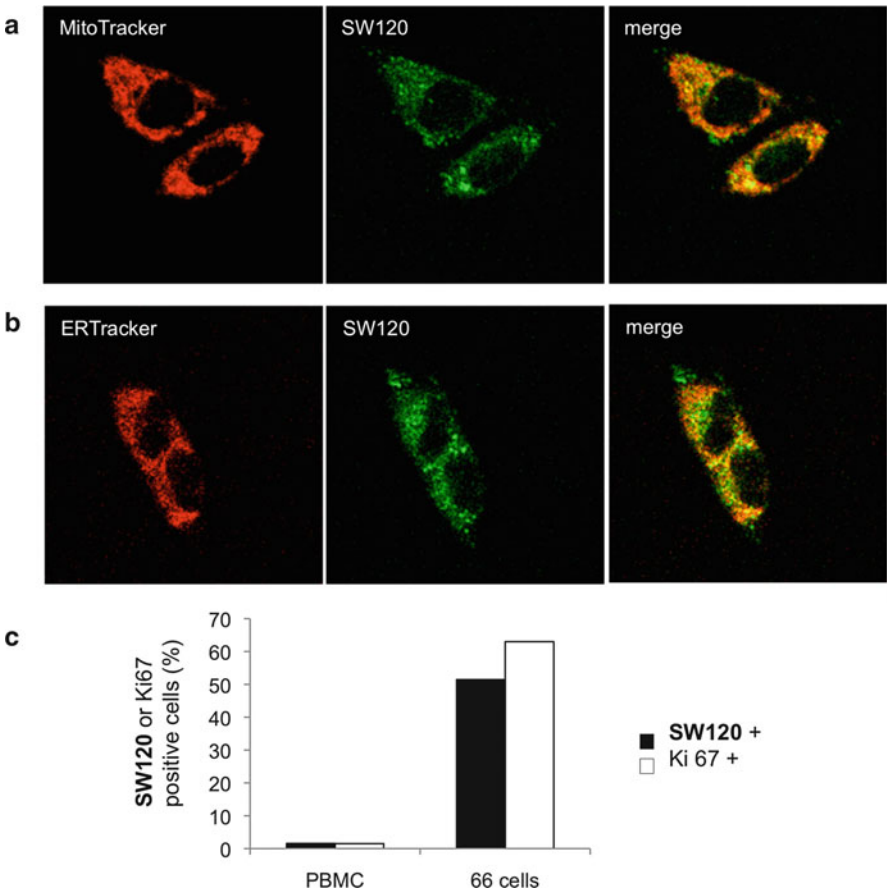

Fig. 4 Subcellular distribution of SW120 in MDA-MB-435 cells with and without MitoTracker (**a**), and ER-Tracker (**b**). Ki-67 expression and SW120 fluorescent intensity in solid mouse breast tumors and peripheral blood mononuclear cells (PBMC) of mice by flow cytometric analysis (**c**)

fluorescent probes based on SV-119, PB28, and the 3,4-dihydroisoquinoline-1-one scaffold are shown in Fig. 3.

The fluorescent probes shown in Fig. 3 have provided valuable information regarding the localization of σ_2 receptors in breast tumor cells. Zeng et al. (2007, 2011) conducted a series of confocal and two-photon microscopy studies in MDA-MB-435 cells incubated with 30 nM K05-138 (confocal microscopy studies) and SW120 (two-photon microscopy studies) and five subcellular organelle markers: the mitochondria tracker, MitoTracker Red CMXRos (20 nM), the endoplasmic reticulum tracker, ER-Tracker™ Red (500 nM), the lysosome tracker, LysoTracker Red DND-99 (50 nM), the nuclear marker, DAPI (300 nM), or the plasma membrane tracker, FM 4-64FX (5 μg/mL). The results showed that SW120 and K05-138 partially co-localized with MitoTracker (Fig. 4a), ER-Tracker (Fig. 4b), LysoTracker, and the plasma membrane tracker,

suggesting that σ_2 receptors may localize in mitochondria, endoplasmic reticulum, lysosomes, and the plasma membrane. The data also showed that SW120 and K05-138 did not co-localize with the nuclear marker, DAPI, suggesting that either the σ_2 receptor does not exist in the nucleus or SW120 does not enter the nucleus. Similar results were obtained for the other σ_2 fluorescent probes (SW116 and SW107) (Zeng et al. 2007, 2011). This subcellular distribution of σ_2 receptors was recently confirmed with confocal microscopy studies by Abate et al. using the fluorescent probes based on PB28 (Abate et al. 2011) and the 3,4-dihydroisoquinoline-1-one scaffold (Niso et al. 2015) (Fig. 3b).

The microscopy studies conducted by Zeng et al. (2007) and Abate et al. (2011) have provided useful information for the interpretation of studies evaluating σ_2 receptor ligands as potential cancer chemotherapeutic agents. Mitochondria are a key organelle to regulate the intrinsic pathway of apoptosis. Apoptotic signals such as UV irradiation or treatment with chemotherapeutic agents cause the release of cytochrome C from the mitochondria and the subsequent activation of caspase-3 leading to an apoptotic cell death (Jiang and Wang 2004). The subcellular localization of σ_2 ligands in mitochondria is consistent with previous studies demonstrating that σ_2 ligands trigger apoptosis in tumor cells by acting on mitochondria (Balakumaran et al. 2009). The endoplasmic reticulum (ER) serves as a dynamic Ca^{2+} storage pool (Berridge 2002). σ_2 selective ligands have been reported to induce transient Ca^{2+} release from the ER, which may be a mechanism for σ_2 ligand-induced cell death (Vilner and Bowen 2000). The presence of the σ_2 fluorescent probes in the ER is consistent with these results. Lysosomal proteases, such as cathepsins, calpains, and granzymes, have been reported to contribute to apoptosis (Chwieralski et al. 2006). Under physiological conditions, these proteases are found within the lysosomes but are released into the cytoplasm upon exposure to cell damaging agents, thereby triggering a cascade of intracellular events leading to cell death. The σ_2 selective ligand siramesine has been reported to cause lysosomal leakage and induce cell death by caspase-independent mechanisms (Ostenfeld et al. 2005, 2008). The localization of fluorescent σ_2 receptor probes in the lysosomes is consistent with the premise that siramesine induces cell death partially by targeting lysosomes to cause lysosomal damage, the release of proteases, and eventually cell death. Evidence has also been reported that σ_2 receptors exist in lipid rafts which are mainly found in the plasma membrane (Gebreselassie and Bowen 2004). Lipid rafts play an important role in the signaling associated with a variety of cellular events including adhesion, motility, and membrane trafficking (Brown and London 1998; Simons and Toomre 2000). The observation that σ_2 fluorescent ligands are co-localized with cytoplasmic membrane markers, and undergo receptor mediated endocytosis, is consistent with their localization in lipid rafts.

Since the σ_2 receptor has been validated as a proliferation marker in cell culture and in solid tumors, it is possible that σ_2 fluorescent probes could preferentially label proliferating cells versus nonproliferating cells and serve as agents to image cell proliferation in vivo. To test this hypothesis, nude mice implanted with murine mammary adenocarcinoma 66 cells (Zeng et al. 2011) were treated with SW120 (50 μg in 100 μL PBS) for 1 h. Both peripheral blood mononuclear cells (PBMC), which are commonly used as controls for nonproliferative cells, and tumor cells

were isolated from the mice. These cells were analyzed by flow cytometry for SW120 uptake and for Ki67 expression, a commonly used proliferation marker. The data showed that PBMC were Ki67 negative, whereas a large portion of the tumor cells were Ki-67 positive (Fig. 4c). The data also showed that PBMC were not labeled by SW120, whereas a portion of the tumor cells were labeled with SW120. The trend of the positive correlation between Ki67 expression and SW120 labeling indicates that the fluorescent probe may possess in vivo selectivity toward proliferating cells versus nonproliferative cells. The data also suggest that σ_2 receptor ligands hold a potential to serve as cancer chemotherapeutics since they may selectively target tumor cells in vivo.

5 In Vivo Imaging Studies of σ_2 Receptors

The conformationally flexible benzamide analog YUN252 has been used in the development of PET radiotracers for imaging the σ_2 receptor status of solid tumors. The first PET radiotracers prepared were the ^{11}C-labeled analogs shown in Fig. 5a (Tu et al. 2005). MicroPET and tumor uptake studies were conducted with [^{11}C]1-4; the most promising analog proved to be [^{11}C]4. Although all four analogs had a high affinity for σ_2 receptors, the optimal lipophilicity of [^{11}C]4 played an important role for the high tumor uptake and suitable signal:normal tissue ratios for imaging (Fig. 5b). These data indicate that both receptor affinity and lipophilicity are important properties for successful receptor-based tumor imaging agents. MicroPET/CT imaging studies in EMT-6 breast tumors show the potential of [^{11}C]4 as a radiotracer for imaging the σ_2 receptor status of breast tumors with PET.

Although [^{11}C]4 demonstrated feasibility in the microPET imaging studies described above, the short half-life of carbon 11 ($t_{1/2} = 20.4$ min) is not ideal for the utilization of PET radiotracers in multicenter clinical PET imaging studies. The longer half-life of ^{18}F ($t_{1/2} = 109.8$ min) compared to ^{11}C places fewer time constraints on tracer synthesis, allows imaging studies to be conducted up to 2 h after injection of the radiotracers, and often results in higher tumor:normal tissue ratios relative to their ^{11}C-labeled analogs. A number of ^{18}F-labeled conformationally flexible benzamide analogs (Fig. 6a) have been evaluated in murine breast tumor models (Tu et al. 2007). The design of these analogs involved replacement of the 2-methoxy group in the benzamide ring with a 2-fluoroethoxy group. The 2-fluoroethoxy- for methoxy-substitution is a common strategy used in the development of ^{18}F-labeled radiotracers. Biodistribution studies in female Balb/c mice bearing EMT-6 tumor allografts demonstrated that all four ^{18}F-labeled compounds had a high tumor uptake (2.5–3.7% ID/g) and acceptable tumor: normal tissue ratios at 1 and 2 h post-i.v. injection. The moderate to high tumor/normal tissue ratios and the rapid clearance from the blood for [^{18}F]ISO-1 and [^{18}F]ISO-2 suggest that these radiotracers are likely the best candidates for imaging of solid tumors with PET. MicroPET imaging studies indicate that [^{18}F]ISO-1 and [^{18}F]ISO-2 are suitable probes for imaging the σ_2 receptor status of solid tumors with PET (Fig. 6b).

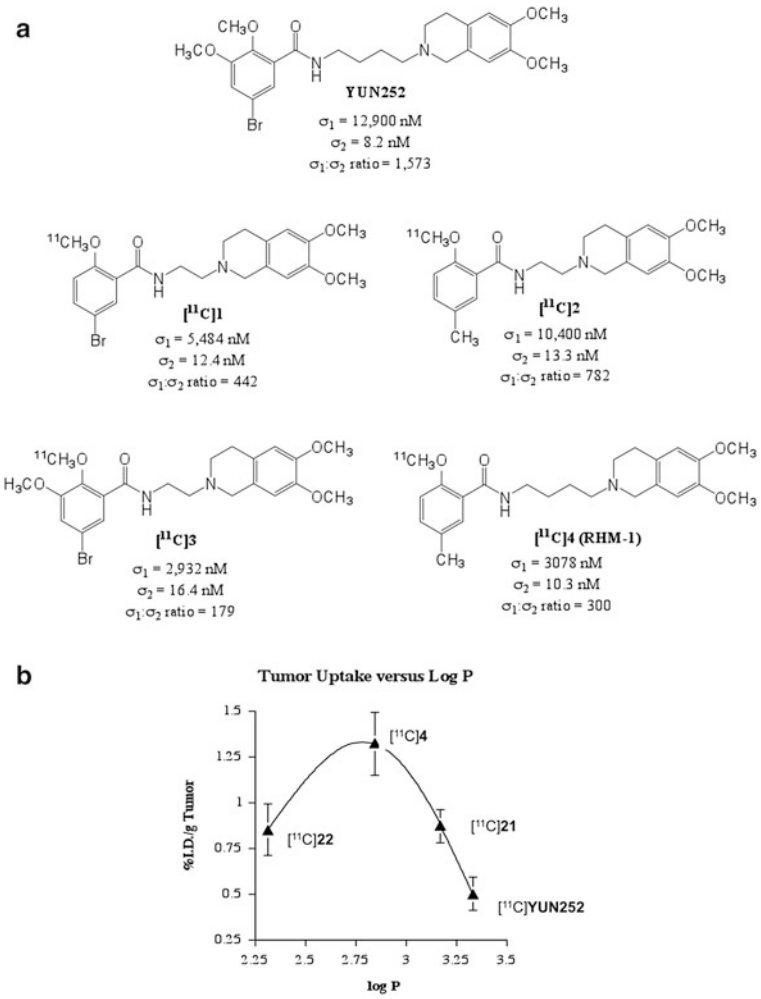

Fig. 5 Structure of YUN252 and ^{11}C-labeled radiotracers, [^{11}C]1–4 (**a**). The relationship between tumor uptake and log P is shown in (**b**)

A number of ^{18}F- and ^{11}C-labeled analogs of the σ_2 receptor ligands developed in the Abate lab have been reported in the literature. The 3,4-dihydroisoquinoline-1-one analog, [^{18}F]5, which can be considered to be a rigid analog of the conformationally flexible benzamide derivatives described above, was found to have a high affinity for σ_2 receptors and an excellent selectivity for σ_2 versus σ_1 receptors (Fig. 6c). This compound was developed as a radiotracer for imaging σ_2 receptors in the CNS; unfortunately, the radiotracer has very low brain uptake, which may be a consequence of it being a substrate for P-glycoprotein. The aminotetralin analog, [^{11}C](*S*)-6, which is structurally related to PB28 and has a good σ_2 affinity and reasonable selectivity versus σ_1 receptors, was evaluated in a similar manner and was found to have a high uptake in

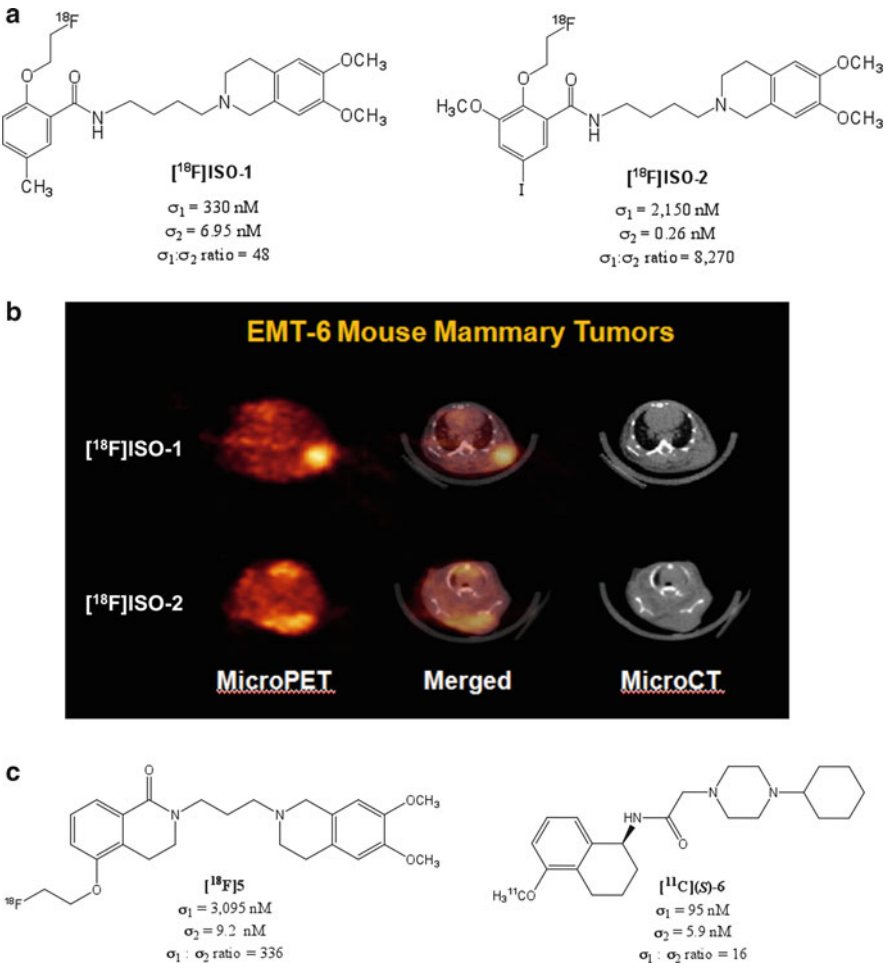

Fig. 6 Structures of [^{18}F]ISO-1 and [^{18}F]ISO-2 (**a**) and microPET imaging studies of these radiotracers in a murine model of breast cancer (**b**). Structures of the PET radiotracers based on **PB28** and the 3,4-dihydroisoquinoline-1-one scaffold (**c**)

rodent brain. Surprisingly, this radiotracer had only a modest uptake in EMT6 mouse breast tumor allografts. However, the tumors used in this imaging study were quite large and likely had regions of tumor necrosis, and possibly quiescence, which could reduce the uptake of a σ_2 receptor imaging agent.

A second technique which could be used in molecular imaging studies of tumors is Single Photon Emission Computed Tomography or SPECT. This technique was the Nuclear Medicine imaging procedure of choice prior to the emergence of clinical PET imaging studies. Although it does not have the prominence it once had, SPECT still represents a sensitive molecular imaging technique and would benefit from the

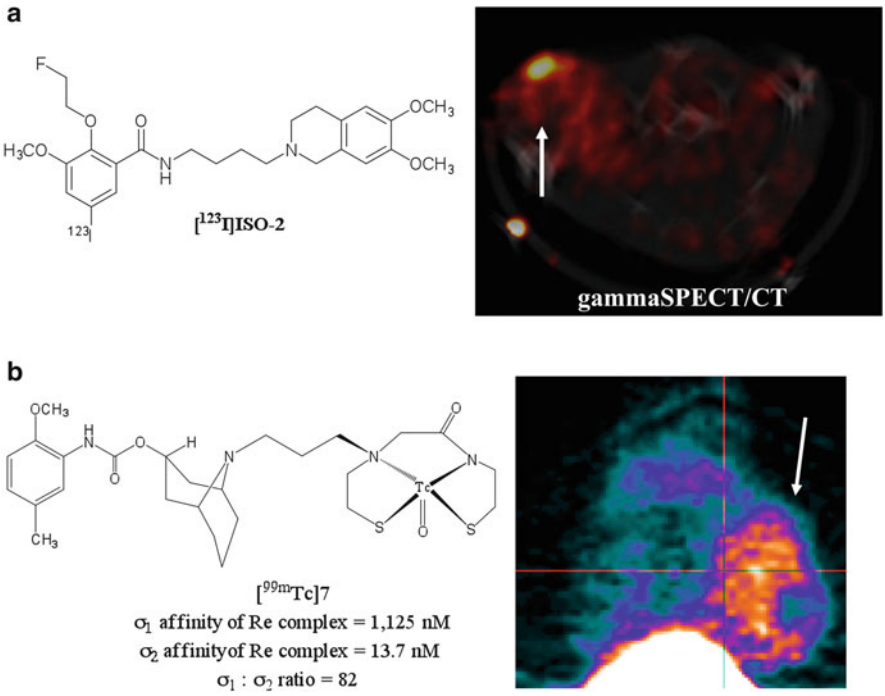

Fig. 7 Structure of [^{123}I]ISO-2 and gammaSPECT imaging in a murine model of breast cancer (**a**). Structure of [^{99m}Tc]7 and microSPECT studies in the same murine model of breast cancer (**b**). The *arrow* points to the tumor in each image

development of receptor-targeting radiotracers for tumor imaging studies. One advantage SPECT has over PET is that it does not require a cyclotron to produce the radionuclides used in the radiolabeling studies; the longer half-lives of the SPECT radionuclides (e.g., 6 h for ^{99m}Tc and 13.2 h for ^{123}I) make it more accessible for delayed imaging studies. A limited number of ^{123}I- and ^{99m}Tc-labeled σ_2 receptor probes have been reported in the literature. The presence of an iodo group in compound ISO-2 indicates that it can be readily labeled with ^{123}I, and gammaSPECT/CT images of [^{123}I]ISO-2 in a murine model of breast cancer indicate that it has potential as an SPECT radiotracer for translational imaging studies (Fig. 7a). A ^{99m}Tc-labeled analog of SV-119 (Fig. 7b) has also been prepared and evaluated in a murine model of breast cancer (Mach et al. 2001). Since there are no stable isotopes of Tc, in vitro binding studies to determine the σ_2 receptor affinity and selectivity versus σ_1 receptors were conducted with the corresponding rhenium (Re) analog. Re-7 was found to have a reasonable σ_2 affinity and good selectivity versus σ_1 receptors. In vivo studies with [^{99m}Tc]7 demonstrated a clear visualization of 66 murine breast tumor xenografts in nude mice (Fig. 7b). These data suggest that [^{99m}Tc]7 may also be a useful radiotracer for SPECT imaging studies of breast cancer patients.

6 The Importance of Imaging Proliferative Status in Cancer Patients

Treatments that specifically target proliferation and produce a cytostatic response are not well evaluated by traditional imaging methods such as MRI, CT, and bone scan (Marinovich et al. 2013). Measures of cancer metabolism using the radiolabeled glucose analog, [^{18}F]FDG, provide information about cancer response to cytotoxic and endocrine agents, but cannot measure proliferation (Avril et al. 2001), proliferation rate, or tumor proliferative status (Shoghi et al. 2013). Many researchers have noted that there is a need for new biomarkers to enable patient selection for new cell-cycle drugs and sophisticated methods need to be developed to measure the effect of these drugs on cellular proliferation (Migliaccio et al. 2014).

An excellent method of imaging cell proliferation would be to develop a tracer that closely mimics the expression of Ki-67, the "gold standard" method for measuring proliferation in histology studies. Ki-67 is expressed in all phases of the cell cycle and has low expression in quiescent tumor cells and senescent normal tissue. While the radiolabeled thymidine analog, [^{18}F]FLT, is well established as a proliferation marker, there are two key features that create the need to find an alternative agent to image proliferation: (1) [^{18}F]FLT has high background uptake in bone marrow, making it impossible to monitor bone metastasis and (2) [^{18}F]FLT is taken up only during S phase, measuring proliferation rate, and not during G1, M, and G2, like Ki-67, which measures proliferative status.

While Ki-67 is a good biomarker for measuring proliferative status in biopsy specimens, there are no small molecules that could be used in the development of PET radiotracers for imaging Ki-67. The σ_2 receptor behaves exactly like Ki-67 and has small molecules that bind with high affinity, allowing PET radiotracer development. Therefore, the σ_2 receptor radiotracers described above provide the only means to date to image the proliferative status of solid tumors with PET.

The only σ_2 *selective* PET radiotracer that has been used in human studies is [^{18}F]ISO-1 (Fig. 6). There is a high correlation between uptake of [^{18}F]ISO-1 and the P:Q ratio of 66 solid mammary tumors (Fig. 8a) (Shoghi et al. 2013). These data indicate that [^{18}F]ISO-1 images both proliferating and quiescent tumor cells. An early validation study in a dichotomous group of patients with head and neck, lymphoma, and breast cancer demonstrated specific uptake in cancer with ability to stratify patients into high or low Ki-67 scores based on [^{18}F]ISO-1 uptake (Fig. 8b) (Dehdashti et al. 2013). The results of this study provided the foundation for an expanded clinical trial in breast cancer patients which is currently ongoing at the University of Pennsylvania. Additionally, bone marrow uptake was at a low level, making this a possible imaging agent for bone metastasis (Fig. 8c), in contrast to what has been reported for [^{18}F]FLT (Mankoff et al. 2005; Shields et al. 1998).

In addition to identifying primary tumors and metastases, imaging the proliferative status with [^{18}F]ISO-1 could be used to guide cancer therapy. An example of this is the therapies targeting the cyclin-dependent kinase 4/6 (CDK4/6). The current indication for the newly approved CDK 4/6 inhibitor, palbociclib, is for primary therapy of ER-expressing metastatic breast cancer, where the addition of palbociclib has been shown to

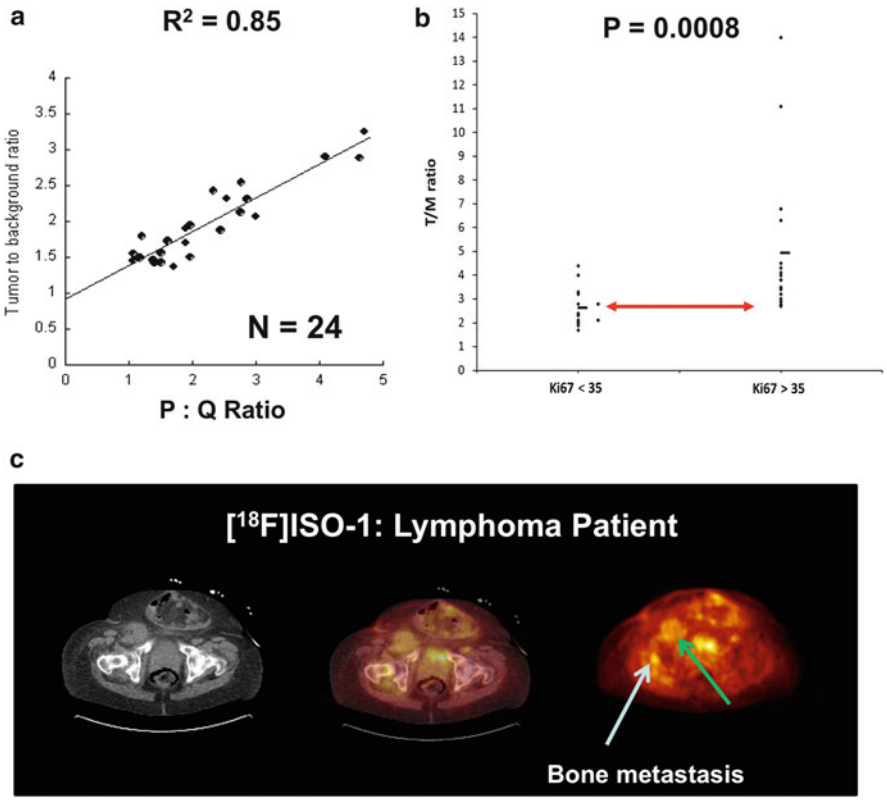

Fig. 8 Correlation of [^{18}F]ISO-1 uptake and P:Q ratio in a murine model of breast cancer (**a**) and with Ki-67 score in a heterogeneous population of cancer patients (**b**). PET imaging studies of [^{18}F]ISO-1 in a patient with lymphoma having a bone metastasis (**c**)

provide an average clinical benefit (Beaver et al. 2015). Currently, the use of palbociclib is guided by the same marker used to direct endocrine therapy, namely the presence of hormone receptors. However, it is difficult to predict which individual patients will benefit from this intervention (Carey and Perou 2015). There is evidence that proliferative measures, like Ki-67, provide an early indication of response to endocrine therapy. In the Immediate Preoperative Anastrozole, Tamoxifen, or Combined with Tamoxifen (IMPACT) trial, pretreatment Ki-67 levels were prognostic for endocrine response and the change in Ki-67 from baseline to 2 weeks after therapy initiation was predictive; in multivariate analysis only the 2-week value was an independent predictor of risk and long-term outcome (Klintman and Dowsett 2015). Therefore, a PET study with [^{18}F]ISO-1 to measure proliferative status is expected to be useful in identifying patients likely to have a favorable response to CDK4/6 inhibitors.

7 Molecular Identification of the σ_2 Receptor

Although the above preclinical and clinical imaging studies have identified the σ_2 receptor as a biomarker for imaging solid tumors, the clinical significance of this protein as a receptor-based marker of cell proliferation has been impeded by the fact that the molecular identity of this protein was not known. The early work of Bowen et al. yielded valuable information regarding the molecular weight of the σ_2 receptor, its localization in lipid rafts, and its involvement as a mediator of cell death (Bowen 2000; Gebreselassie and Bowen 2004). However, these studies did not lead to either the sequencing of the σ_2 receptor protein or the identification of the gene encoding this protein.

In 2011, the Mach group reported their work to determine the molecular identity of the σ_2 receptor. This group developed a strategy to utilize a σ_2 selective photoaffinity probe, WC-21, to irreversibly label σ_2 receptors in rat liver membrane homogenates (Xu et al. 2011). WC-21 contains an azide moiety for the photoaffinity tagging of the protein and an FITC group for protein visualization (Fig. 9a). WC-21 exhibits high binding affinity for sigma-2 receptors (Ki = 8.7 nM) and relatively low binding affinity for sigma-1 receptors (Ki > 4,000 nM). The rat liver membrane homogenates were photolabeled with 100 nM WC-21 and then separated by gel electrophoresis. The western blot analysis using anti-FITC antibodies showed that WC-21 labeled a protein band at ~24 kD. Labeling of this protein band with WC-21 could be blocked by DTG and haloperidol, which are ligands with high affinities to σ_1 and σ_2 receptors, as well as RHM-1, which is the σ_2 ligand. These data suggest that WC-21 labeled proteins are σ_2 receptors. Proteomic studies of the protein in the ~24 kD band labeled by WC-21 identified progesterone receptor membrane component 1 (PGRMC1). A review of the literature revealed a number of similarities between PGRMC1 and sigma-2 receptors: (1) both PGRMC1 and σ_2 receptors are cancer biomarkers and therapeutic targets, (2) both are found in microsomal membranes and have similar subcellular localization, (3) both are associated with cytochrome P-450 proteins, and (4) progesterone binds to both PGRMC1 and σ_2 receptors with the modest affinity. It is also important to note that the molecular weight of the protein sequence of the PGRMC1 is 21.4 kDa, which is virtually identical to the 21.5 kDa of the σ_2 receptor identified previously (Hellewell et al. 1994). Therefore, PGRMC1 was chosen for further validation.

Receptor binding studies showed that the PGRMC1 ligand, AG-205, and the known σ_2 ligands, DTG, siramesine, SV119, and WC-26, readily displaced σ_2 radioligand, [^{125}I]RHM-4, binding in HeLa cell membrane homogenates. Knockdown of PGRMC1 using a PGRMC1-specific siRNA reduced the binding of [^{125}I]RHM-4 to HeLa cells (Fig. 9a), whereas the overexpression of PGRMC1 in HeLa cells increased the binding of [^{125}I]RHM-4. Knocking down PGRMC1 in HeLa cells decreased caspase-3 activation induced by WC-26, suggesting that σ_2 ligand-induced caspase-3 activation is mediated by PGRMC1. Taken collectively, the results of these studies suggest that the PGRMC1 protein complex is the putative σ_2 receptor binding site.

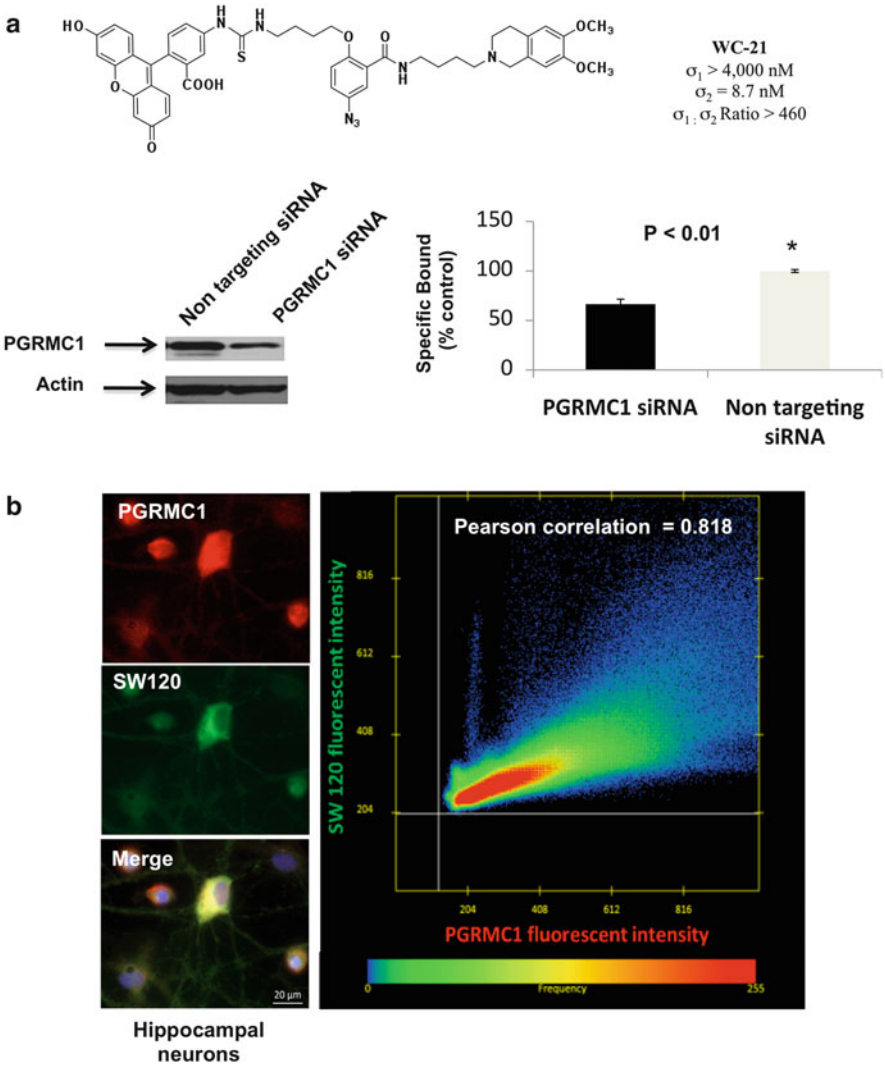

Fig. 9 Photoaffinity labeling of the PGRMC1 using the σ_2 receptor photoaffinity probe WC-21 (**a**). Co-localization of the PGRMC1 with the σ_2 fluorescent probe SW120 (**b**)

Recently, Izzo and colleagues reported the link between the σ_2 receptor and PGRMC1 during the discovery and development of small molecule therapeutics targeting beta amyloid (Aβ) 1-42 oligomers for treating Alzheimer's disease (AD) (Izzo et al. 2014). Soluble oligomers of Aβ have been recognized to be early and key intermediates in AD related synaptic dysfunction. Soluble Aβ oligomers caused synaptic dysfunction and impaired performance in memory tasks. Alterations in membrane trafficking induced by Aβ oligomers are believed to mediate synaptic dysfunction. By screening a library of

central nervous system drug-like small molecules for their abilities to reverse Aβ-induced membrane trafficking deficit in primary neurons, the lead compounds were identified. These compounds can displace Aβ oligomer binding to synaptic puncta, and prevent and reverse Aβ oligomer-induced synapse loss in primary neuronal culture, as well as reverse the memory loss in mouse models of AD. In order to identify the molecular target of the lead compounds, these compounds were examined for their activities to displace radioligand binding to receptors or for their effects on enzyme activities for 100 receptors and enzymes in the central nervous system. As a result, these compounds were found to be σ_2 receptor ligands. Since PGRMC1 has been reported to be associated with the σ_2 receptor, validation of PGRMC1 as a potential molecular target of the σ_2 ligands was conducted. The data showed that knockdown of PGRMC1 with increasing doses of siRNA decreased Aβ binding to neurons up to 91% in a dose dependent manner. Incubation of PGRMC1 antibody against the C-terminal amino acids 185–195 of PGRMC1 in live cells for 30 min significantly reduced Aβ oligomer binding to hippocampal and cortical neurites. These data suggest that PGRMC1 is a key mediator in Aβ oligomer-induced synaptic dysfunction. That both σ_2 ligand treatment and knockdown of PGRMC1 can reduce Aβ oligomer binding to neurons strongly supports the concept that PGRMC1 or its closely associated proteins contain the σ_2 receptor binding site.

In another recent report, the Mach group demonstrated the positive correlation between PGRMC1 protein expression and σ_2 fluorescent probe binding activity in rat hippocampal cell culture (Zeng et al. 2015). In this study, the PGRMC1 protein levels were examined in rat primary cultures of neurons, astrocytes, oligodendrocytes, and microglia cells by immunohistochemistry. The σ_2 receptor binding activities of SW120, a σ_2 fluorescent probe, were also examined in the aforementioned cell types. The data showed that the PGRMC1 is expressed in all brain cell types but with different expression levels. The expression level of PGRMC1 in neurons is consistently higher than that in astrocytes, oligodendrocytes, and microglia. Similarly, SW120 binding activity is also high in neurons and relatively low in astrocytes, oligodendrocytes, and microglia. In order to study whether PGRMC1 and SW120 co-localize in the cells, double staining of a rat hippocampal cell mixture for PGRMC1 with anti-PGRMC1 antibodies and the σ_2 receptor with SW120 was performed. The results showed that PGRMC1 and SW120 partially co-localized in the same subcellular organelles of cells and PGRMC1 protein levels and SW120 binding activity were highly correlated (the Pearson correlation coefficient is 0.818) (Fig. 9b). These results are consistent with our previous report that the PGRMC1 protein complex is the putative σ_2 receptor binding site.

Two recent reports have challenged the concept that the σ_2 receptor binding site resides within the PGRMC1 protein complex. Abate and colleagues (Abate et al. 2015) stably silenced PGRMC1 with shRNA targeting PGRMC1 or overexpressed PGRMC1 in human MCF7 adenocarcinoma cells. Western blot analyses showed that PGRMC1 protein level was reduced by about 80% in PGRMC1 knockdown cells, whereas PGRMC1 protein level is increased by about twofold in PGRMC1 overexpressed cells. Scatchard analyses with radioligand [^{3}H]DTG showed that the σ_2 receptor densities in wild-type, PGRMC1 knockdown and PGRMC1 overexpressed cells are essentially the same. They also showed that AG205, a known

PGRMC1 ligand, did not displace [^{3}H]-DTG in the competition binding assay in rat liver membranes (Ki > 10,000 nM). The data suggest that PGRMC1 is not the binding site of DTG and does not affect the DTG binding site either.

In another report, the Ruoho group knocked out or overexpressed PGRMC1 using CRISPR/cas9 inhibition technology in a mouse motor neuron-like cell line, NSC34 cells (Chu et al. 2015). Western blot analysis showed that PGRMC1 expression was reduced by more than 90% in knockdown cells and dramatically increased in overexpression cells. Scatchard analysis using [^{3}H]DTG showed that the maximal σ_2 receptor densities (Bmax) and the binding affinities (Kd) of [^{3}H]DTG were similar in the wild-type, PGRMC1 knockout and overexpression cells. In addition, using a σ_2 photoaffinity probe [^{125}I]-iodoazido-fenpropimorph ([^{125}I]IAF) developed in this group (Fontanilla et al. 2008), they showed that the intensities of the [^{125}I]IAF photolabeled protein band (~18 kDa) on an SDS gel were similar in the wild-type and PGRMC1 knockout cells, and this band could be protected by DTG but not (+)-pentazocine, a specific σ_1 ligand. These results suggested that PGRMC1 and the σ_2 receptor are distinct binding sites. However, the molecular weight of the protein labeled by [^{125}I]IAF (~18 kDa) is significantly lower than the 21.5 kDa molecular weight of the σ_2 receptor reported previously (Hellewell et al. 1994).

One possible explanation for these discordant findings is that since the PGRMC1 is a protein complex containing one or more partner proteins, the ligands used in the above studies label different members of the PGRMC1 protein complex. That is, the azide group of WC-21 comes in contact with PGRMC1, but the DTG binding site resides on one of the partner proteins making up the PGRMC1 protein complex. Studies aimed at identifying the different partner proteins that bind to the PGRMC1 and form the σ_2 receptor binding site should clear up the discordant observations described above.

8 Conclusions

The σ_2 receptor continues to be an important protein in the field of tumor biology. The high expression of this receptor in proliferating versus quiescent breast tumors indicates that the σ_2 receptor is an important clinical biomarker for determining the proliferative status of solid tumors using the functional imaging techniques PET and SPECT. The σ_2 receptor fluorescent probes identified the subcellular localization of σ_2 receptors using confocal and two-photon microscopy techniques, and this information has proven useful in identifying the mechanism of action of σ_2 receptor ligands as cancer chemotherapeutics. The full utility of the σ_2 receptor in the diagnosis, prediction of therapeutic response, and treatment of cancer will rely on additional studies clarifying the functional relationship between the σ_2 receptor binding site and PGRMC1, and the functional role of this protein complex in normal and tumor cell biology.

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